BUTYRATE REGULATION OF GENE EXPRESSION IN COLONIC EPITHELIAL CELLS

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For my Mother and Father, who always
taught me to strive to be the best I could be

Tamdiu discendum est, quamdiu vivas

Contents

List of Figures

List of Tables

List of Abbreviations

ACF	aberrant crypt foci
APS	ammonium persulphate
ATP	adenosine triphosphate
BBMV	brush border membrane vesicle
BSA	bovine serum albumin
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CH_2Cl_2	dichloromethane
CRC	colorectal cancer
CTP	cytosine triphosphate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DEAE	diethylaminoethyl
DEME	Dulbecco's modified eagle's medium
dH_2O	distilled water
ddH_2O	deionized distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
DPP IV	dipepidyl-peptidase IV
DTT	dithiolthreitol
EDTA	ethylenediaminetetraacetic acid
FAP	familial adenomatous polyposis
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid
HFB	sodium heptafluorobutyrate
HNPCC	hereditary non-polyposis colorectal cancer
HRP	horse radish peroxidase
IAP	intestine alkaline phosphatase
INT	iodotetrozolium succinate
LMV	luminal membrane vesicle
MES	2-(N-morpholino)ethanesulphonic acid

MCT1	monocarboxylate transporter 1
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger RNA
NaBut	sodium butyrate
NaHFB	sodium heptafluorobutyrate
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
PMSF	phenylmethanesulphonyl fluoride
PNPM	*p*-nitrophenol-α,D-mannoside
PNPP	*p*-nitrophenolphosphate
ppm	parts per million
PPP	poly(2,5-bis(3-sulphonatopropoxy)-1,4-phenylene disodium salt
PVDF	polyvinyldiflouride
RNA	ribonucleic acid
SARKOSYL	sodium lauroyl sarcosinate
SDS	sodium dodecyl sulphate
SSC	sodium citrate, sodium chloride
TBS	tris buffered saline
TCA	tricarboxylic acid cycle
TEMED	N,N,N′,N′-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TEA	triethylamine
TEAB	triethylammonium bicarbonate
TEAH	triethylammonium salt
TLC	thin layer chromatography
TMS	tetramethylsilane
U	unit
UV	ultra violet

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Abstract

Dietary Plant-derived fibre and resistant starch are fermented in the colon by the colonic microflora to short chain fatty acids, acetate, propionate and butyrate. Butyrate is a major source of energy for colonocytes and plays a critical role in maintaining colonic tissue homeostasis by regulating the expression of genes associated with proliferation, apoptosis and differentiation of colonic epithelial cells. Some examples of these genes include, Cyclin D1, CD1 (a key positive regulator of cell cycle progression, downregulated by butyrate) p21 (a cell cycle inhibitor, upregulated by butyrate), intestinal alkaline phosphatase, IAP (a marker of differentiation, upregulated by butyrate), bak (upregulated by butyrate) and bcl-x_L (down-regulated by butyrate), the latter functionally opposing butyrate responsive members of bcl2 family important to the regulation of cell death by apoptosis.

Previous work in the laboratory had demonstrated that:

- Butyrate is transported across the colonic luminal membrane by the monocarboxylate transporter isoform 1 (MCT1).
- Butyrate regulates MCT1 expression at both transcriptional and post-transcriptional levels.
- Using the technique of short interfering RNA to knock down the expression of MCT1, and hence butyrate transport, in cultured colonic epithelial cell lines i) MCT1 is the major route for the entry of butyrate into the cell and ii) the inhibition of MCT1, and butyrate transport, abolishes the butyrate dependant regulation of CD1, p21 and IAP, indicating that butyrate entry via MCT1is required for regulation of these genes. However inhibition of MCT1 had no effect on butyrate modulation of bak and bcl-x, implying a potential presence of a butyrate sensor on the colonic luminal membrane to initiate signalling pathway involved in regulation of bak and bcl-x_L.

Work presented in this thesis:

To identify further the mechanism involved, at the membrane level, for butyrate regulation of gene expression in the colon, a membrane impermeable butyrate analogue was synthesised. The procedure involved coupling poly(ethylene glycol) $_{600}$ (PEG_{600}), a membrane impermeable molecule with an approximate molecular weight of 600 kDa to the C4 position of butyric acid. Disodium $PEG_{600}\alpha,\omega$-dibutyrate was fully characterised by 1H and 13C NMR, mass spectrometry and IR spectroscopy, to assess the structure and purity.

Disodium $PEG_{600}\alpha,\omega$-dibutyrate, was used to treat two colonic epithelial cell lines, AA/C1 and HT-29. For control, cells were treated with sodium butyrate. Techniques of northern blotting, real-time PCR and western blotting were performed to determine the mRNA and protein abundance of IAP, p21, MCT1, bak and bcl-xL in cells treated either with disodium PEG600α,ω-dibutyrate or sodium butyrate. Disodium $PEG_{600}\alpha,\omega$-dibutyrate had no effect on the regulation of p21, IAP, MCT1 and bcl-xL. However, disodium $PEG_{600}\alpha,\omega$-dibutyrate treatment resulted in the up-regulation of bak mRNA and protein to the same extent as observed with butyrate treatment. This indicates that butyrate regulation of bak is not dependent on entry of butyrate into the cell. In contrast butyrate regulation of IAP, p21 and MCT1, demands butyrate entry into the cell.

Transport studies using radiolabelled [U-^{14}C] butryate into colonic luminal membrane vesicles (LMV) were performed i) to characterise butyrate transport, ii) to determine if heptafluorobutyrate (HFB, a non-metabolisable analogue of butyrate), is transported by MCT1, and iii) to assess potential and type of inhibition of butyrate transport by HFB. Treatment of colonic cells with HFB had no effect on the expression of number genes that required butyrate entry into the cell, indicating that the metabolism of butyrate may be required for the regulation of these genes.

CHAPTER 1

Introduction

1.1 Anatomy of the large intestine

1.1.1 Gross anatomy of the large intestine

The large intestine consists of approximately 1.5 to 1.8 m section of the alimentary tract that extends from the ileum to the anus; with a diameter of approximately 6 cm. The large intestine is attached to the posterior abdominal wall by the mesocolon, a double layer of peritoneum, and has four main sections: cecum, colon, rectum and anal canal.

The cecum is a small pouch about 6 cm long and is divided from the ileum of the small intestine by the ileocecal sphincter. Connected to the cecum is the colon which is divided into the ascending, transverse, descending and sigmoid colon. The ascending colon has an approximate length of 12 to 20 cm and

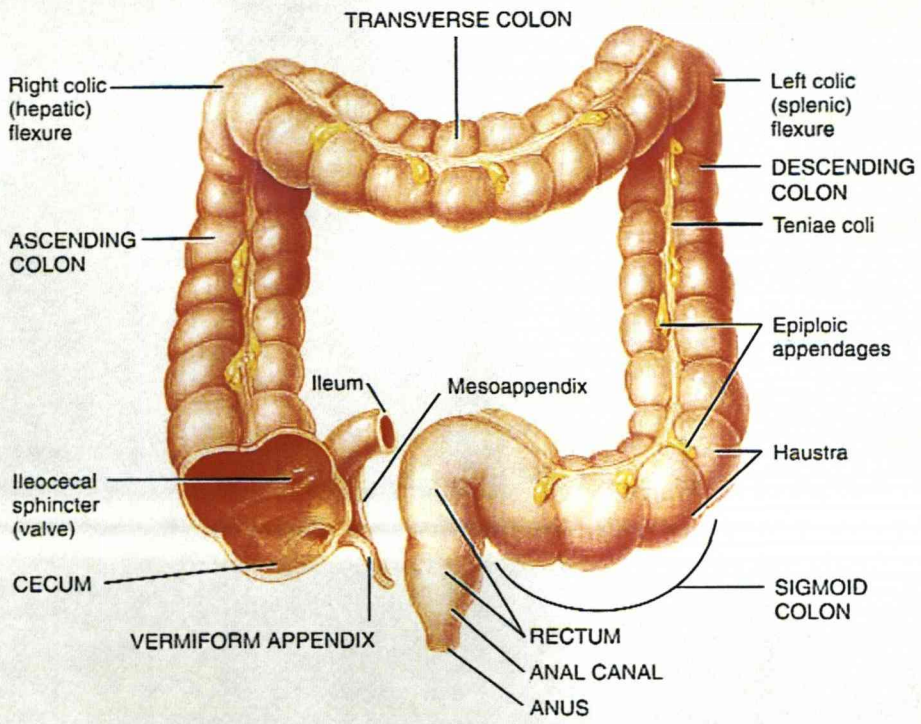


Figure 1.1 Gross anatomy of the large intestine

Adapted from Tortora & Derrickson, (2006).

rises from the cecum up the right side of the abdomen to the liver where it turns left to form the right colic (hepatic) flexure. The colon proceeds across the abdomen above the small intestine as the transverse colon, which has an approximate length of 50 cm, curving beneath the spleen at a 90 ° angle as the left colic (splenic) flexure. Here the colon continues descending down the left side of the abdomen as the descending colon with an approximate length of 25 cm, passing the left kidney and terminating at the iliac crest. The sigmoid colon, so called due to its S-shape begins near to the iliac crest from the descending colon, runs medially to the midline of the abdomen where it terminates as the rectum. The rectum extends for approximately the last 20 cm of the alimentary tract descending anterior to the coccyx. The last 2 – 3 cm of the rectum forms the anal canal with the opening of the anal canal to the exterior being the anus. The anus terminates with an internal and external anal sphincter (Tortora & Grabowski, 2003).

1.1.2 The colonic wall

Although the large intestine is larger in diameter than the small intestine, there are no villi present and so the intestinal wall is thinner. The wall of the colon is made up from four layers: mucosa, submucosa, muscularis externa and serosa. The mucosa is subdivided into: epithelium containing epithelial, goblet, stem and endocrine cells; lamina propria which contains connective tissue to support the epithelial layer; and muscular mucosae that consists of longitudinal and circular layers of smooth muscle.

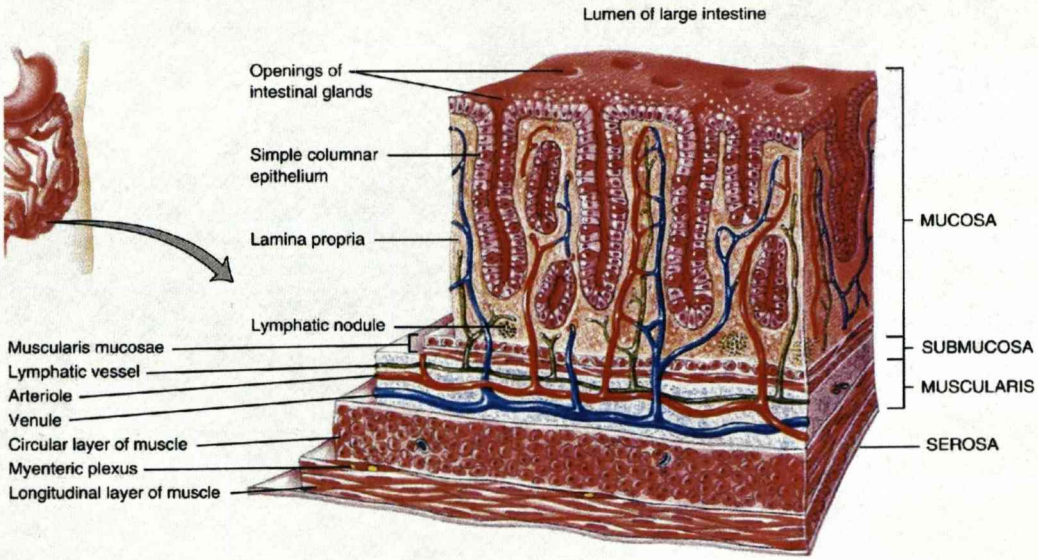


Figure 1.2 Illustration of the human colonic wall

Adapted from Tortora & Grabowski, (2003).

The submucosa consists of supportive connective tissue; intertwined within the submucosa are lymphatic and blood vessels. The muscularis consists of an external layer of longitudinal smooth muscle that surrounds an internal layer of circular smooth muscle. Parts of the longitudinal smooth muscle are thickened forming three conspicuous longitudinal bands called the taniae coli that run the length of the large intestine. The taniae contract to gather the colon into a series of pouches called haustra, giving the colon its characteristic appearance. The serosa consists of a continuous sheet of squamous epithelial cells and forms part of the visceral peritoneum. Small pouches of visceral peritoneum filled with fat are attached to the teniae coli and are referred to as epiploic appendages (Shamsuddin, 1990).

1.1.3 The colonic epithelium

The colonic epithelium runs the length of the colon and consists of a great number of tubular glands called *glands of Lieberkuhn* or *crypts*. The crypts are very abundant and stretch from the bottom to the top of the mucosa layer, are about 0.5 mm deep in most of the colon and are surrounded by lamina propria (Singh & Binder, 2001). At the base of the crypt are 1 to 4 stem cells whose daughter cells differentiate into goblet, enteroendocrine and epithelial cells. The epithelial cells and goblet cells have a half-life of approximately 6 days; the enteroendocrine cells have a longer half-life (Christensen, 1991). The cells migrate up the crypt axis from the base to the crypt peak, differentiating in the upper portion and after 3 to 4 days undergo apoptosis and are shed into the

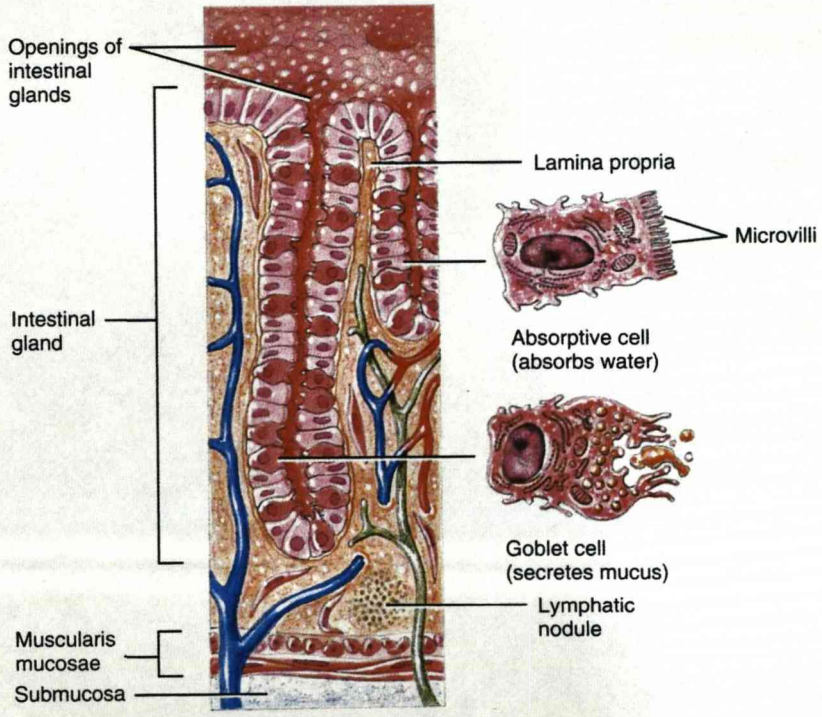


Figure 1.3 Illustration of a human colonic crypt

Adapted from Tortora & Grabowski, (2003).

lumen (Kim *et al.*, 1994; Hall *et al.*, 1994).

The epithelial cells, or colonocytes, are the principle cells in the colonic epithelium. Colonocytes are tall cylindrical cells that contain microvilli on their luminal membranes and have basally positioned nuclei and mitochondria. They are joined at the lumen poles by junctional complexes to adjacent colonocytes forming a barrier between the lumen of the large intestine from the paracellular spaces between the colonocytes. The colonocytes are responsible for the absorption of water and nutrients across the epithelia from the lumen into the blood stream at the basal pole of the colonocyte (Singh & Binder, 2001).

The goblet cells, so called due to their goblet shape, are mucous producing cells. They also contain a basally located nucleus and secrete mucous into the lumen which helps the passage of faecal material through the colon. The enteroendocrine cells are the least abundant and sit deep within the epithelium. Enteroendocrine cells often display an apical finger that extends towards the lumen. They are responsible for secretions into the lamina propria at the base of the crypts (Colony, 1989).

1.2 Functions of the colon

The colon once thought to have little function, actually plays a significant role in the digestion and absorption of ingested food. Approximately 600 – 1500 ml of matter enters the colon daily compared to only 200 ml of faeces exiting (Macfarlane & Cummings, 1991). The majority of fluid is absorbed by the proximal portion of the colon while the rectum is essentially non-absorbing, being relatively impermeable to sodium and water (Phillips, 1969). The large intestines functions to reabsorb water; absorb nutrients and vitamins released by the intestinal microflora; absorption and secretion of inorganic ions;

compact the contents of the intestine into faeces; and the storage and defecation of faeces (Marieb, 2003).

1.2.1 Absorption and secretion of electrolytes and water

The colon is responsible for the reabsorption of water, the secretion of bicarbonate, chloride, potassium and protons; as well as the absorption and secretion of sodium and chloride ions through a multitude of ion channels and transporters.

The human colon absorbs 0.5 – 1.5 litres of water daily, which is coupled to sodium absorption (Singh & Binder, 2001). The majority of water absorption is accounted for by passive diffusion, transported paracellularly in response to osmotic gradients (Phillips, 1969); mechanisms are needed to create the osmotic gradients as the osmolarity of the faeces is greater then the plasma (Sladen, 1971).

The mammalian colon absorbs sodium against a significant electrochemical gradient and can scavenge to retain sodium at times of sodium depletion. (Singh & Binder, 2001). The capacity of the small intestine to absorb sodium and chloride is limited, however the human colon absorbs 90 % of the sodium and chloride that enters the cecum, preventing diarrhoea and sodium depletion (Devroede & Phillips, 1969). Patients with ileostomies present with chronic sodium depletion and dehydration, highlighting the role of the colon in sodium and water absorption (Clarke *et al.*, 1967). Sodium is absorbed in the colon through two pathways. A sodium conductive pathway that accounts for 40 % sodium absorption through epithelial sodium channels (Dudeja *et al.*, 1994b); and an electroneutral Na/H exchange that accounts for 60 % sodium absorption (Dudeja *et al.*, 1994a). Once inside the colonocytes, sodium is excluded via the Na^+,K^+-ATPase across the basolateral membrane.

Chloride absorption in the human colon is linked to bicarbonate secretion (Sladen, 1971), while chloride secretion is linked to the basolateral electroneutral Na-K-2Cl transporter (Singh & Binder, 2001;Sandle, 1998). Chloride transport is electroneutral, with Cl/HCO_3 exchange coupled to Na/H exchange (Foster *et al.*, 1986). Bicarbonate is produced by intracellular hydration of CO_2 by carbonic anhydrase producing carbonic acid which rapidly degrades to bicarbonate (Cummings, 1984). Cl/HCO_3 exchange accounts for a portion of chloride absorption with sodium stimulated chloride absorption also occurring (Davis *et al.*, 1983). A portion of paracellular chloride absorption also takes place (Geibel, 2005).

The colon is responsible for maintaining the potassium balance within the body, especially when kidney function is impaired (Cummins, 1984). Potassium is secreted and absorbed in the distal colon (Devroede & Phillips, 1969), where potassium ions are brought into the cell from the blood by the Na^+,K^+-ATPase, on the basal side of the cell and secreted into the lumen by potassium channels (Cummings, 1984). Potassium ions are absorbed from the lumen by the H^+,K^+-ATPase (Horisberger, 2001).

1.2.2 The colonic microflora

The large intestine, as mentioned above, plays host to a large colony of bacteria. The colon is inoculated after birth and the adult human colon can contain up to 400 different species of bacteria, covering 50 genera (Macfarlane & Cummings, 1991; Topping & Clifton, 2001); a gram of caecal content may contain up to 2 billion organisms (Chapman, 2001). *Bacteroides*, *Bifidobacterium* and *Enterococcus* (Bugaut & Bentejac, 1993; Pryde *et al.*, 2002) are present with *eubacterium*, *lactobacillus* and *clostridium* being predominant, while Bacteroides account for 30 % of the total bacterial dry

mass (Macfarlane & Cummings, 1991; Hill, 1995a). Table 1.1 outlines the bacteria genera, and their numbers present per gram faeces.

On average bacteria make up approximately 40 – 55 % of stool matter with approximately 15 g of bacteria being expelled per day by defecation for individuals consuming Western style diets (Macfarlane & Gibson, 1995; Topping & Clifton, 2001). There is an excess of 10^{11} bacterial cells per dry weight of intestinal content (Bugaut & Bentejac, 1993), that averages 10^{13} - 10^{14} organisms per colon with a metabolic activity of that of the liver (Hill, 1995a). Bacterial proliferation occurs more significantly in the proximal colon where there is fresh intake of food from the small intestine; bacterial proliferation in the distal colon is not so prominent (Macfarlane & Cummings, 1991; Topping & Clifton, 2001).

Diet has been found to influence the microflora of rat, mice and ruminants but in man it is accepted that diet does not have a significant effect on the microflora population. However persons living on vegetarian, Japanese and Western-type diets do exhibit changes in key populations like bifidobacteria and eubacteria (Macfarlane & Cummings, 1991). The microflora utilise a wide range of organic matter for food consisting of exfoliated epithelial cells, glycoproteins, pancreatic secretions, lysed bacterial cells and dietary fibre (Bach Knudsen *et al.*, 2003; Bugaut & Bentejac, 1993; Hill, 1995a).

1.2.3 Fermentation of indigestible material by the colonic microflora

Starch, the common storage of glucose in cereal grains, potatoes and some root potatoes is digested by α-amylases present in the small intestine. The remaining polysaccharides that are resistant to digestion by α-amylase, are termed non-starch polysaccharides (NSP), or more commonly, dietary fibre.

NSP represents the plant cell wall materials including the polysaccharides cellulose, hemicellulose and pectin; storage polysaccharides, inulin and guar; and plant gums and exudates (Cummings, 1981; Kritchevsky, 1995). More recently the importance of resistant starch has been noted. Resistant starch (RS) is the term used to describe starch polysaccharide that due to food processing escapes digestion in the small intestine. RS was discovered through investigations into the amount of NSP found in cooked foods. The data showed increased amounts of NSP after cooking than the raw foods. This increase in NSP was found to be a glycan (referred to as RS) that upon investigation was measured as glucose and accounted for the increase in NSP after cooking (Englyst & Cummings, 1987).

Table 1.1 Bacterial flora of the adult human colon

Genus of bacteria	Log_{10} (number per g faeces)
Non-sporing anaerobic bacteria	
Bacteroides spp	10 - 11
Bifidobacterium spp	10 - 11
Eubacterium spp	9 - 11
Propionibacterium spp	9 - 11
Veillonella spp	5 - 8
Sporing anaerobic bacteria	
Clostridium spp	5 - 10
Bacillus spp	3 - 7
Microanaerobic bacteria	
Lactobacillus spp	7 - 9
Streptococcus spp	7 - 9
Enterococcus spp	5 - 7
Facultative organisms	
Escherichia spp	7 - 9
Other enterobacteria	5 - 9

Adapted from Hill, (1995b).

Currently there are four categories of resistant starch:

Physical inaccessibility; foods which are inaccessible to pancreatic enzymes. These include whole or partly milled grains and seeds and foods which in its physical form presents a barrier i.e. corn, peas, beans and rice.

Ungelatinised starch granules; starch in its natural form has a granule structure that depends on the chain length of the starch. During cooking the granules are gelatinised, or breakdown, allowing access to α-amylase. Foods eaten raw, such as bananas, contain large amounts of ungelatinised starch. Three types of granule are known, distinguished by their X-ray diffraction pattern.

Retrograde starch; heated foods on cooling or drying produce starch granules. The granule produced depends on the temperature of cooking and includes potatoes, rice and maize.

Chemically modified; Processed foods that are chemically modified (e.g. esterified starches) where the modification interferes with the amylolytic activity of the digestive enzymes.

(Macfarlane & Cummings, 1991; Perrin *et al.*, 2001; Topping & Clifton, 2001)

NSP and RS can be divided into soluble and insoluble depending on there solubility in aqueous solutions; soluble fibres are widely fermented while insoluble fibres are poorly fermented (Wolever, 1994). Typically 50 - 60 g carbohydrate are fermented in the human colon per day for individuals on a common European diet (Bergman, 1990). This represents an abundant food source for the resident microflora.

Figure 1.4 illustrates the major pathways of carbohydrate fermentation and the products formed in the human colon. The polysaccharides, oligosaccharides and disaccharides contributed by fibre and RS are metabolised by the colonic microflora; saccharides are hydrolysed to their constituent sugars by extracellular enzymes before being fermented (Wolin, 1994). Undigested

proteins or endogenous pancreatic secretions are also fermented, as too are the sugars lactose, raffinose and starchylose (Cummings *et al.*, 1987; Topping & Clifton, 2001)

Mucus secreted from the small intestine contains mucins that are acidic glycoproteins containing galactose, hexosamines and fucose. Mucins also enter the large intestine where they are fermented (Macfarlane & Cummings, 1991); 4 to 6 g of pancreatic enzymes and 2 to 3 g small intestinal mucins may enter the colon every day (Macfarlane & Gibson, 1995). In man the principle end products of fermentation are short-chain fatty acids (SCFA), the gases, CH_4, CO_2 and H_2, plus some heat (Bugaut & Bentejac, 1993; Hill, 1995b; Pouteau *et al.*, 2003).

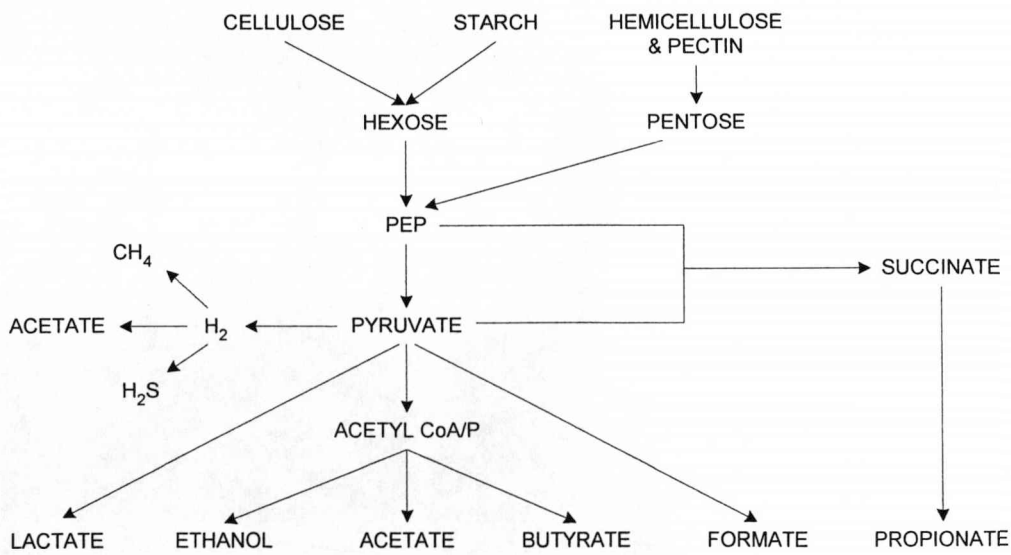


Figure 1.4 Major pathways of carbohydrate fermentation in the human large intestine

Overview of the major pathways of NSP and RS fermentation in the human large intestine and the principle products formed.
Adapted from Macfarlane & Gibson, (1995).

1.3 Dietary fibre, resistant starch and colonic health

Fibre and RS have several promoting effects on the health of the colon. Primary investigations into the promoting effect of dietary fibre centred on the roughage model. The hypothesis presumed that dietary fibre acted as a bulking agent in the large intestine, reducing the transit time of faecal matter and so to the amount and time that the colonic mucosa is exposed to carcinogens (Burkitt, 1971). However, as previously discussed, it is now known that the colonic microflora are able to utilise dietary fibre for their energy requirements with short-chain fatty acids (SCFAs) being the down-stream products of fermentation and are the source of extensive research. SCFAs have widened the previous hypothesis surrounding the protective effect of dietary fibre and RS on colon carcinogenesis.

Addition of fibre to the diet has been found to increase stool bulk, reducing transit time through the colon, diluting and binding of potential carcinogens, reduce the production of secondary bile acids and lowing of the colonic pH (Kritchevsky, 1995; Peters *et al.*, 2003). However, it is difficult to identify a single mechanism by which dietary fibre modifies colon carcinogenesis (Bugaut and Bentejac, 1993). The increase in stool bulk is thought to come about by increased proliferation of the colonic microflora through increased nitrogen metabolism within the colon (Hill, 1995a). The production of SCFAs reduces the colonic pH and the production of secondary bile acids (Bingham *et al.*, 2003). Secondary bile acids are said to be colon tumour promoters; reduction of secondary bile acids results from the reduction in 7-α-dehydroxylase activity, the enzyme responsible for the conversion of primary to secondary bile acids. (Le Leu *et al.*, 2003). The addition of fibre to the diet of rats has been found to stimulate crypt cell proliferation. Furthermore, the

addition of fibre to germ-free rats showed no stimulatory effect indicating the requirement of fermentation by the colonic microflora (McCullogh *et al.*, 1998). Impaired fermentation of dietary fibre and butyrate production leading to reduced sodium absorption has been implicated in the causes of diarrhoea (Livesey & Elia, 1995). Interactions between dietary fibre and other nutrients also have an effect on the health of the colon. Whether these are synergistic additive effects have yet to be decided (Kritchevsky, 1995).

1.4 Short-chain fatty acids

1.4.1 Production of SCFAs

SCFAs are small alkyl carboxylic acids ranging in length from 1 to 6 carbons and are produced in large quantities, approximately 80 to 130 mmol per kg of luminal contents. Greater numbers of SCFA are produced in the cecum and proximal colon, owing to a greater carbohydrate source and lower pH, (pH 6.3) than the distal colon (pH 6.6) (Macfarlane & Cummings, 1991). The principle SCFAs produced by far, are acetate, propionate and butyrate, which account for 85 to 95 % of total SCFA production (Bergman, 1990). The branched chain fatty acids isobutyrate and isovalerate are found, but in much smaller quantities. Production of acetate, propionate and butyrate is not equal with the ratio of acetate:propionate:butyrate averaging approximately 57:22:21 (Bugaut & Bentejac, 1993; Cummings, 1994); the actual ratio of acetate, propionate and butyrate depends on the food source.

Table 1.2 lists several sources of NSP and RS with the ratio of acetate, propionate and butyrate produced after fermenting for 24 hours in *in vitro* incubation systems inoculated with fresh human faecal flora. Resistant starch, wheat bran and oat bran produced high amounts of butyrate whereas xylan,

pectin and guar gum produce relatively little amounts of butyrate upon fermentation (Bach Knudsen *et al.*, 2003; Kritchevsky, 1995).

Table 1.2 SCFA fermentation ratios obtained from dietary fibres

Substrate	% Acetate	% Propionate	% Butyrate
Resistant starch	41	21	38
Starch	50	22	29
Oat bran	57	21	23
Wheat bran	57	15	19
Cellulose	61	20	19
Guar gum	59	26	11
Ispaghula	56	26	10
Pectin	75	14	9

SCFA ratios obtained from dietary fibres in *in vitro* incubation systems inoculated with fresh human faecal flora.
Adapted from Kritchevsky, (1995).

1.4.2 Metabolism of SCFAs

SCFA are rapidly absorbed from the lumen by the colonic epithelium; with approximately 90 % of SCFA absorbed and only 10 % residing in the faeces (Hill, 1995a; Macfarlane & Cummings, 1991). SCFA are transported across the colonocytes into the blood where they are transported through the circulation to be utilised by different tissues depending on the SCFA. The transepithelial transport of butyrate is significantly lower than for acetate or propionate with the majority of butyrate being utilised by the colonocytes as their preferred food source (Demigne & Remesy, 1994). Butyrate metabolism within the colonocyte is preferred over propionate and acetate (Cummings *et al.*, 1987) with more than 70 % of oxygen consumption by isolated colonocytes due to butyrate metabolism *in vitro* (Roediger, 1980). Moreover butyrate oxidation in

colonocytes is not suppressed in the presence of other energy-providing substrates, while the presence of butyrate will suppress the oxidation of other SCFAs including acetate (Fitch & Fleming, 1999). Butyrate is metabolised in the mitochondria to acetyl-CoA via the β-oxidation cycle. Mitochondrial acetyl-CoA is then used in the TCA cycle for the production of ATP, and the synthesis of the ketone bodies 3-hydroxybutyrate and acetoacetate via ketogenesis (Livesey & Elia, 1995), However this is a minor route for butyrate metabolism (Bugaut, 1987). Acetyl-CoA yielded from butyrate metabolism is also used as a precursor for *de novo* lipid synthesis in the cytosol (Macfarlane & Cummings, 1991). However, butyrate utilisation in *de novo* lipogenesis is secondary to acetate utilisation in rat colonocytes (Zambell *et al.*, 2003).

The majority of acetate is transported across the colonic epithelium where it enters the bloodstream and is metabolised by the peripheral tissues. Acetate is metabolised in brain and muscle tissue to CO_2 (Livesey & Elia, 1995) and is metabolised by the liver during ketogenesis. Hepatic vein SFCA concentrations are lower than the portal blood indicating take up of SCFA by the liver (Cummings *et al.*, 1987; Livesey & Elia, 1995), with propionate and butyrate being almost exclusively taken up by the liver (Bergman, 1990; Bach Knudsen *et al.*, 2003). Propionate absorbed by the liver is principally metabolised to succinyl-CoA; conversion with acetyl-CoA yields oxaloacetate which enters the TCA cycle (Stephen, 1994). Butyrate taken up by the liver is metabolised to acetyl-CoA via β-oxidation, which is oxidised to CO_2 during the TCA cycle (Demigne & Remesy, 1994).

1.4 Transport of SCFAs

SCFAs are weak acids with pKa's around 4.5. The luminal environment of the colon has an approximately neutral pH resulting in significant dissociation of the SCFAs; 99 % of SCFAs are in their anionic form. Table 1.3 shows the pKa values for SCFAs with carbon numbers of 1 to 6. Transport of SCFA is accompanied with increased luminal bicarbonate and is thought to be coupled to Na/H exchange, which accounts for the stimulated sodium absorption and bicarbonate secretion that accompanies the absorption of SCFAs (Sellin, 1999; Cummings, 1981). There are currently two widely accepted models of SCFA transport in the colonic mucosa, non-ionic diffusion and carrier-mediated transport; both are discussed below.

1.4.1 Non-ionic diffusion

The first investigations into transport of SCFAscentred on the idea of non-ionic diffusion of SCFAs across the luminal membrane. Early studies proposed SCFA diffusion linked to sodium absorption (Smyth & Taylor, 1958). Ionic diffusion of the Na^+ or K^+ salt of the SCFA has also been proposed that accounted for a small portion of SCFA transport (Ruppin *et al.*, 1980). As the SCFA are present in the lumen in their anionic form, protonation to the corresponding acid must occur prior to non-ionic diffusion.

There are two mechanisms for the production of protons for the protonation of SCFAs. 1) HCO_3^- and H^+ are produced in the lumen by the colonic microflora from the hydration of CO_2; 2) intracellular secretion of H^+ into the lumen by the Na^+/H^+ exchanger or H^+/K^+ exchanger could also supply the necessary protons (Ruppin *et al.*, 1980; Bugaut, 1987).

As the degree of SCFA dissociation is so great, a high degree of protonation would be required to account for the rapid transport of SCFAs. To explain this, an unstirred water layer adjacent to the microvilli was proposed. An unstirred water layer refers to a layer of water adjacent to a biological membrane that is not at equilibrium with the bulk of the surrounding water. Unstirred water layers exist adjacent to cell membranes in the lumen of the human colon and contribute significantly to the absorption of molecules through the membrane (Dietschy *et al.*, 1971). An unstirred water layer has been shown to exist in rat colon that contains a pH microclimate (Titus & Ahearn, 1992). This microclimate maintains a different pH to that of the central part of the lumen, at which the SCFA are protonated to a higher degree (Bugaut, 1987; Barry& Smyth 1960). However, there are several observations that can not be explained by this theory. SCFA are still absorbed in the absence of Na/H exchange and bicarbonate. The degree of protonation and absorption of SCFA is too small to account for the rapid influx of SCFA from the lumen. Finally a decrease in luminal pH would be expected with rapid transport of SCFA due to

Table 1.3 pK_a values for SCFAs

Carbon No.	Formula	IUPAC name	common name	pK_a
1	HCOOH	Methanoic	Formic	3.55
2	CH_3COOH	Ethanoic	Acetic	4.56
3	C_2H_5COOH	Propanoic	Propionic	4.67
4	C_3H_7COOH	Butanoic	*n*-Butyric	4.63
4	$(CH_3)_2CHCOOH$	2-Methylpropanoic	*iso*-Butyric	4.63
5	C_4H_9COOH	Pantanoic	*n*-Valeric	4.64
5	$(CH_3)_2CHCH_2COOH$	3-methylbutanoic	*iso*-Valeric	4.58
6	$C_5H_{11}COOH$	Hexanoic	*n*-Caproic	4.63

SCFA IUPAC and common names (with acid omitted) and their pK_a at 25 °C, ionic strength 0.1 mol/l. Adapted from Fukushima, (1995).

the movement of protons into the lumen, however this is not seen (Titus & Ahearn, 1992).

1.4.2 Carrier-mediated transport

Carrier-mediated transport can be characterised with the observation of the substrates being transported against a chemical or electrochemical gradient, saturation kinetics, energy requirements for the carrier operation and competitive substrate inhibition (Bugaut, 1987; Titus & Ahearn, 1992). Studies carried out on guinea-pig isolated colon in Ussing chambers showed bicarbonate dependent SCFA transport that was explained as a SCFA/HCO3- exchanger (Engelhardt *et al.*, 1989). Studies carried out using luminal membrane vesicles isolated from rat distal colon, characterised an anion exchange process in which SCFAs were transported into the vesicles in exchange for bicarbonate (Mascolo *et al.*, 1991; Stein *et al.*, 1995). SCFA transport into pig and human colonic luminal membrane vesicles has also been investigated (Harig *et al.*, 1996; Ritzhaupt *et al.*, 1998a). Transport studies of SCFAs using luminal membrane vesicles showed that SCFA transport demonstrated saturation kinetics, was stimulated by an outward bound HCO3- gradient and inhibited by other SCFAs (Harig *et al.*, 1996; Ritzhaupt *et al.*, 1998a; Stein *et al.*, 1995); all characteristics of a carrier-mediated transport system. Furthermore, transport of butyrate into Caco-2 cells - a colonic adenocarcinoma cell line that forms viable monolayers of polarised cells - showed butyrate transport that demonstrated saturation kinetics, was stimulated by low pH, inhibited by other SCFAs and demonstrated SCFA/HCO_3 exchange (Stein *et al.*, 2000). Transport of propionate into human ileal brush-border membrane vesicles has also been shown to follow saturation kinetics,

enchanced by intra-vesicle HCO_3^- and low pH and was inhibited by other SCFAs (Harig *et al.*, 1991).

1.4.3 Identification of SCFA transport by MCT1

Work carried out by Rizthaupt *et al.*, (1998a) on butyrate transport into porcine and human colonic luminal membrane vesicles, characterised butyrate transport as a pH-activated electroneutral anion exchange mechanism. Transport of butyrate into colonic LMVs showed saturation kinetics and was stimulated by an outward directed anion gradient in the order of butyrate > bicarbonate > propionate > chloride. Furthermore, butyrate transport into colonic LMVs was enhanced at low pH but was not dependent on a pH gradient, with butyrate transport being inhibited by other SCFAs, namely propionate and lactate (Ritzhaupt *et al.*, 1998a). The protein responsible for butyrate transport into porcine and human colonic LMVs was determined to be the monocarboxylate transporter isoform 1 (MCT1). Northern blotting for MCT1 showed the presence of a single transcript of 3.3 kb in porcine and human colonic tissue. Western blotting using an antibody to MCT1 gave a single band of 40 kDa in purified and well-characterised human and porcine colonic LMVs that was enriched 15- to 20-fold. Furthermore, injection of MCT1 mRNA into oocytes of *Xenopus laevis*, demonstrated transport of butyrate and L-lactate into the oocytes that was inhibited by butyrate, L-lactate and pyruvate (Ritzhaupt *et al.*, 1998b).

1.5 Monocarboxylate transporter (MCT) family

1.5.1 The MCT gene family

To date the MCT family consists of 14 members, with all members sharing at least 20 – 25 % amino acid sequence homology (Hediger *et al.*, 2004). Hydrophobicity plots predict 10 – 12 α-helical trans-membrane (TM) domains for the MCT family with a large hydrophilic loop between TM domains 6 and 7, and the N- and C-terminus located in the cytoplasm (Enerson & Drewes, 2003); this topology has been confirmed experimentally for MCT1 in rat erythrocytes (Poole *et al.*, 1996) and is illustrated in figure 1.5. The TM domains have high sequence homology between family members and across species. However, the large intracellular loop between TM domains 6 and 7 differs greatly between members (Halestrap & Meredith, 2004).

1.5.2 Identification of MCT1

MCT 1 was the first member of the MCT family to be identified. MCT1 was discovered in 1992 by Kim et al., (1992) as the transporter responsible for the movement of mevalonate across the plasma membrane in a mutant line of Chinese hamster ovary (CHO) cells. At the time, the transporter was given the name Mev. Sequence analysis of Mev cDNA and wild type cDNA showed a point mutation in the Mev cDNA conferring a change from phenyalanine (wild-type) to cysteine (Mev) (Kim *et al.*, 1992). The wild-type protein was found to transport mevalonate to a much smaller degree and exhibited the classical properties of the monocarboxylate transporter (MCT) that was

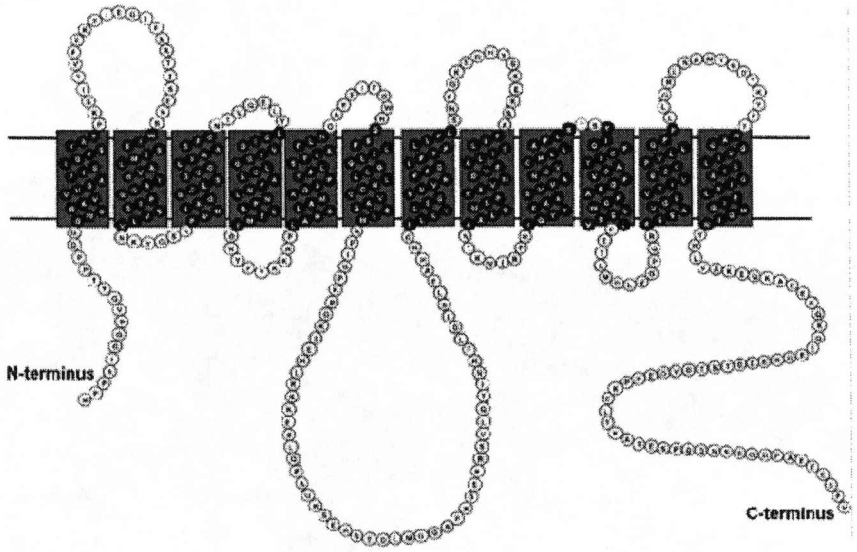


Figure 1.5 Predicted topology of MCT1

Adapted from Halestrap & Price, (1999).

responsible for the transport of lactate into erythrocytes. After sequencing and functional testing the transporter was given the name MCT1 (Garcia *et al.*, 1994a). MCT1 has the gene symbol SLC16A1, is a member of the SCL16 monocarboxylate cotransporter gene family, and human MCT1 is mapped to locus 1p13.2-p12 on chromosome 1 (Garcia *et al.*, 1994b).

1.5.3 Characterisation of MCT1

MCT1 has a wide tissue distribution; to date MCT1 has been found expressed at the level of mRNA in mouse, rat and human, skeletal muscle, heart, kidney, brain, small intestine, colon, testis and red blood cells (Dubouchaud *et al.*, 2000; Halestrap & Price, 1999; Lambert *et al.*, 2002; Philp *et al.*, 2003; Price *et al.*, 1998). Equally, the substrates available for transport by MCT1 are wide ranging. Short chain (C2-C5) unbranched aliphatic monocarboxylates such as acetate and butyrate and substituted C2 or C3 monocarboxylates such as

pyruvate, L-lactate, acetoacetate and D-β-hydroxybutyrate are all transported (Halestrap & Meredith, 2004). Expression of MCT1 in rat skeletal muscle has 83 % and 86 % homology with human MCT1 nucleotide and amino acid sequences respectively (Jackson *et al.*, 1995). MCT1 has been found to transport L-lactate in erythrocytes (Halestrap & Price, 1999) and ketone bodies and L-lactate in sheep ruminal epithelium (Muller *et al.*, 2002). MCT1 catalyses net transport of one carboxylate with a proton, exchange for one monocarboxylate with another (Halestrap & Meredith, 2004) or exchange for a monocarboxylate with bicarbonate (Ritzhaupt *et al.*, 1998b).

1.5.4 Characterisation of other MCTs

Human MCT2 (SLC16A2) shares 49 % amino acid sequence homology with MCT1 (Lin *et al.*, 1998). MCT2 transports pyruvate (Lin *et al.*, 1998; Garcia *et al.*, 1995) and L-lactate (Broer *et al.*, 1999) that is stimulated at low pH (Broer *et al.*, 1999; Lin *et al.*, 1998). The expression of MCT3 (Human gene name SLC16A8) has been localised to the retinal pigment epithelium in rat, mouse and chicken (Philp *et al.*, 2003) and in the choroid plexus epithelium in mouse brain (Philp *et al.*, 2001). MCT3 shares 43 % amino acid homology with MCT1, transports lactate and pyruvate and is inhibited by α-cyano-4-hydroxycinnamate, a known MCT inhibitor (Yoon *et al.*, 1997). MCT4 (Human gene name SLC16A3) is expressed in mouse neural retina (Philp *et al.*, 2003). MCT4 has been shown to catalyse the proton-linked transport of L-lactate (Halestrap & Price, 1999) and is up regulated in skeletal muscle after exercise; that is consistent with it being an L-lactate transporter in this tissue (Dubouchaud *et al.*, 2000).

Information on MCT5 – MCT8 (Human gene name SLC16A4 - SLC16A6) is limited to expression analysis with MCT5 – MCT8 being expressed in various

tissues throughout the human body (Price *et al.*, 1998). TATI is the only MCT that does not transport monocarboxylates and was identified as a member of the MCT superfamily through amino acid sequence homology. Human TAT1 (SCL16A10) transports aromatic amino acids in a sodium- and proton-independent manner. The remaining family members MCT9, MCT11, MCT12, MCT14 and MCT15 (SCL16A9, SCL16A11 – 15) were identified through searching of the human genome and EST databases and there is currently no expression analysis or characterisation data available for these MCTs (Halestrap & Meredith, 2004).

1.5.4 Regulation of colonic MCT1

Human colonic MCT1 was found to be regulated by butyrate but not acetate and propionate; butyrate was demonstrated to up-regulate MCT1 in a concentration- and time-dependent manner (Cuff *et al.*, 2002). This regulation involved transcriptional and post-transcriptional processes. Butyrate up regulates the transcription of MCT1 and stabilises the MCT1 transcript, which in turn increases the transcript half-life (Cuff *et al.*, 2002). MCT1 has been localised to the luminal membrane in human colon (Lambert *et al.*, 2002). Using in situ hybridisation and northern and western blotting MCT1 expression in human colon has been shown to decline in the colon during development from normality to malignancy (Lambert *et al.*, 2002).

1.6 In vivo and In vitro effects of Butyrate

1.6.1 Butyrate effects *in vivo*

There have been a great number of studies investigating the effects of SCFAs, in particular butyrate, *in vivo*. It is generally accepted that butyrate increases colonic mucosal proliferation and reduces apoptosis *in vivo* (Macfarlane & Cummings, 1991; Lupton, 1995).

In vivo investigations are difficult to carry out and control, especially in human subjects and much work has centred on animal studies. Animals fed a high fibre diet have higher rates of colonic mucosal proliferation than animals fed elementary diets. This increase is abolished in germ free animals indicating fermentation of fibre and the down stream products are important (Johnson, 1995). Feeding of fibres promoting stable butyrate production to rats increase the length of the mucosa and the depth of the crypts in the caecum and colon. This effect was only seen with fibres known to produce high levels of butyrate. Furthermore, the high butyrate-producing fibres decreased the rate of aberrant crypt foci in rates preceding injection with azoxymethane (Perrin *et al.*, 2001).

Direct instillation of SCFAs into caecectomised rats fed a fibre-free diet stimulated increased mucosal mass and DNA content. Moreover an increase was also seen after colonic installation with butyrate to a similar level as installation with total SCFAs (Johnson, 1995). Perfusion of individual SCFAs into the gastrointestinal (GI) tract of rats has shown a dose-dependent stimulatory effect on epithelial cell production rates in the jejunum and distal colon (acetate only showed an effect in the colon) in the order of butyrate > propionate > acetate (Sakata, 1995).

Feeding of high amylase cornstarch, a form of resistant starch, to rats showed increased production of butyrate, increased faecal weight, reduced pH and increased apoptosis (Le Leu *et al.*, 2003). Apoptosis has been also shown to

be linked to butyrate. Perfusion studies in Ussing chambers using guinea-pig colonic tissue showed increase apoptosis, and in particular increased *bax* (a proapoptotic gene) expression after 45 minutes post withdrawal of butyrate (Hass *et al.*, 1997; Luciano *et al.*, 1996).

Intervention studies in humans have shown that dietary wheat bran supplements, that are known to raise colonic luminal butyrate levels, can inhibit DNA synthesis and epithelial cell proliferation in the rectal crypts of patients with a resected colon or colorectal cancer (Clausen, 1995). Perfusions of butyrate into the colon of patients with ulcerative colitis has been shown to reduce inflammation and the associated symptoms (Johnson, 1995; Young & Gibson, 1995). Overall, conditions that reduce the production of SCFAs, especially butyrate, can depress epithelial cell proliferation in the large and small intestine. These include bypass surgery, parental nutritional feeding, feeding of a substrate free diet and germ free conditions. The result is depressed mitotic activity and mucosal atrophy in the colon mucosa (Sakata, 1995; Young & Gibson, 1995).

1.6.2 Butyrate effects *in vitro*

Salts of butyrate act on abnormal and transformed cells to bring about a change to a more normal phenotype. Treatment of cultured cells with butyrate is associated with decreased cell growth, bringing about differentiation and increasing apoptosis (Lupton, 1995). The *in vitro* observations seen with butyrate treatment of cultured cell lines are discussed in more detail below.

1.6.2.1 Butyrate and proliferation

Cdks are serine/threonine kinases that require binding of their cyclin partner for activation; the exception being cdk5, which is activated by the non-cyclins p35 and p39 (Loyer *et al.*, 2005). Different cyclin-cdk complexes are activated at different points within the cell cycle and cyclins have to be destroyed in order for the cell to exit mitosis (Murray, 2004). Progression through G_1 requires the D-type and E-type cyclins and their respective cyclin-dependent kinases (Diehl, 2002). Cyclins D1, D2 and D3 bind to cdk4 and cdk6 while cyclin E1 and E2 bind with cdk2 (Sherr & Roberts, 2004). D-type cyclin-cdk complexes are involved in the G_1/S checkpoint; activation leads to phosphorylation and activation of the retinoblastoma protein (pRb), the so called gate keeper of the G_1 phase, which in turn dissolves complexes of pRb with members of the E2F family of transcription factors and associated chromatin-modifying enzymes. The downstream result is activation of the genes required for DNA synthesis and key steps of cell proliferation (Deshpande *et al.*, 2005; Fu *et al.*, 2004). Cyclin-cdk complexes are negatively regulated by the CIP/KIP and INK family of inhibitors. CIP/KIP members include $p21^{WAF1/Cip1}$ (p21) and $p27^{KIP1}$ (p27), while INK members include $p16^{INK4a}$ (p16) and $p19^{INK4c}$ (p19) (Sherr, 1996). p21 and p27 inhibit CD/cdk4 kinase, CD/cdk5 kinase, CE/cdk2 kinase and CA/cdk2 kinase activities to varying degrees (Siavonshian et al., 2000; Sherr, 1996); p21 is the main inhibitor of the CD/cdk4 and CD/cdk6 complexes (Coradini *et al.*, 2000).

The addition of butyrate to cultured cells inhibits proliferation, leading to an arrest of cell growth, which in many cases involves arrest early on in the G_1 phase of the cell cycle (Kruh *et al.*, 1995). See Table 1.4 for a list of genes that are modulated by butyrate. Treatment with sodium butyrate (NaBut) also

suppresses cell proliferation and colony formation in agarose (Lupton, 1995). Butyrate reduces proliferation in HT-29, Caco-2 and SW-1116 colonic epithelial cells with the largest inhibition of proliferation in HT-29 cells, an adenocarcinoma cell line, resulting in 76 % inhibition achieved with 2 mM NaBut. The anti-proliferative effect was similar in Caco-2 cells but stronger in SW-1116 cells, a differentiated cell line, indicating that inhibition is independent of the differentiation state of the cells (Siavoshian *et al.*, 1997). Butyrate-modulated arrest in HT-29 cells is time- and concentration-dependent, occurs prior to the Cyclin E restriction point and is associated with an increase in the number of cells in G_0/G_1 phase of the cell cycle (Coradini *et al.*, 2000; Iacomino *et al.*, 2001). Butyrate modulates the expression of many regulators of the cell cycle. The cyclin-dependent kinase inhibitors p16, p19 and p27 are all up-regulated by butyrate in HT-29 cells (Iacomino *et al.*, 2001); moreover p27 along with p21 was found to be up-regulated, in response to butyrate treatment in Caco-2 cells, in a dose- and time-dependent manner (Litvak *et al.*, 1998).

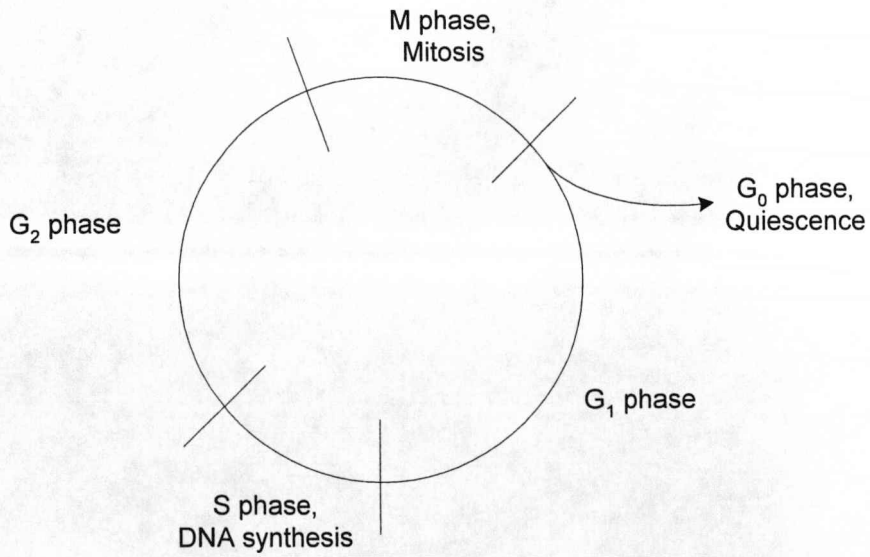


Figure 1.6 Schematic illustrating the cell cycle

Butyrate induces p21 in HT-29 and Caco-2 cells as early as 2 and 3 hours respectively upon butyrate treatment (5-20 mM) that lasts for 48 hours (Orchel *et al.*, 2003). However Coradini et al., (2000) found that while a concentration of 1 mM NaBut induced p21, higher doses above 4 mM down-regulated p21 in HT-29 cells. Butyrate negatively modulates the expression of p53 in HT-29 cells in a dose-dependent manner; p53 is a regulator of p21 transcription indicating that butyrate modulation of p21 is linked to p53 expression (Coradini *et al.*, 2000). Butyrate induces p21 through the $p21^{WAF1/Clip1}$ promoter at Sp1 sites in human colonic adenocarcinoma WiDr and oteosarcoma MG63 cell lines at Sp1 sites -82 to -77 and -69 to -64, relative to the transcription start site. The Sp1 site at -82 to -77 was found to strongly activate p21 while the Sp1 site at -69 to -64 was partially responsible. MG63 cells contain a p53 rearrangement, therefore indicating that the activation of the promoter was independent of p53; however, it should be noted that these cells, are not colon derived (Nakano *et al.*, 1997). The relationship between butyrate anti-proliferative effect and p21 was further supported by Hinnebusch *et al.*, (2000). The authors treated a p21-deleted HCT-116 colonic adenocarcinoma cell line with NaBut and showed no reduction in proliferation. The wild type HCT-116 p21-containing cell line did respond to NaBut treatment, indicating that p21 is required for the butyrate mediated response in HCT-116 cells (Hinnebusch *et al.*, 2002).

Butyrate-induced cell cycle arrest has also been linked to CKII expression. CKII is a serine/threonine protein kinase that requires phosphorylation for activation and regulates transcription factors such as Jun and Myc. Moreover, CKII is vital for cell cycle progression in yeast *Saccharomyces cerevisiae*. Treatment of NaBut to HT-29 cells significantly down-regulates CKII activity at

the post-translational level indicating that butyrate may modulate CKII expression through changes in its phosphorylation state (Russo *et al.*, 1997).

Table 1.4 Butyrate-induced modulation of genes associated with proliferation, differentiation and apoptosis

Positively modulated	Negatively modulated
Proliferation	
p16	p53
p19	CB1
p21	CD1
p27	Cdx2
CD3	CkII
Differentiation	
Glycoprotein synthesis	
Carcino-embryonic antigen	
DPP IV	
IAP	
Apoptosis	
Bak	Survivin
FAST	Bcl-x_L
Caspase-1	Bcl-x_S
Cspaspe-3	Bcl-2

The intestinal *Cdx2* homeobox gene encodes an intestinal transcription factor that is a key regulator of intestinal mucosal homeostasis by inhibiting cell proliferation and inducing differentiation. Butyrate modulates the expression of Cdx2 mRNA and protein through increased transcriptional activity of the *Cdx2* promoter in a time- and concentration-dependent manner (Domon-Dell *et al.*, 2002).

CD1 reaches maximal expression during G_1 phase and is a key regulator of G_1/s progression (Kong *et al.*, 2000). Furthermore, CD1 is frequently associated with human malignancies (Diehl, 2002). Butyrate was found to positively modulate the expression of CD3 and negatively modulate the expression of CD1 and CB1 in HT-29 and HCT-116 cells (Hinnebusch *et al.*, 2002; Siavoshian *et al.*, 2000) Butyrate modulated CD1 at level of mRNA but

not the protein, while CD3 protein was up-regulated by butyrate but not the CD3 mRNA (Siavoshian *et al.*, 2000).

1.6.2.2 Butyrate and differentiation

Butyrate has been found to induce differentiation in a number of non-cancerous cells promoting erythropoiesis and keratinocyte differentiation. (Lupton, 1995) Moreover butyrate treatment to cultured cells increases net production of glycoprotein synthesis, carcino-embryonic antigen (Johnson, 1995), dipeptidyl-peptidase (DPP) IV (Siavoshian *et al.*, 1997) and intestinal alkaline phosphatase (IAP) (Kruh *et al.*, 1994), all markers of differentiation (see table 1.4). The regulation of carcino-embryonic antigen by NaBut involves transcriptional and post-transcriptional mechanisms (Saini *et al.*, 1990). Treatment of NaBut can stimulate mucin production of cultured mucosa obtained from cancer patients (Johnson, 1995), while IAP is up-regulated by butyrate in HT-29 and Caco-2 cells (Ding *et al.*, 2001). Butyrate induced differentiation in HT-29 is linked to p27 expression. HT-29 cells that overexpressed p27 had a marked increase (2-3 fold) in sensitivity to induction of differentiation with 2 mM NaBut (Yamamoto *et al.*, 1999). Yamamoto *et al.*, (1999) used IAP expression as a marker for differentiation.

1.6.2.3 Butyrate and apoptosis

Apoptosis, or programmed cell death, is a naturally occurring process that occurs in all tissues and has an essential role in maintaining tissue homeostasis throughout the body. Cells undergoing apoptosis show characteristics of shrinkage and blebbing of cell membranes, chromatin condensation and DNA fragmentation. Moreover these characteristics are very

distinct from cells undergoing necrosis (Renehan *et al.*, 2001; Thomson, 2001). Apoptosis is principally mediated by a family of cysteine protease caspases that are constitutively present in most cell types. Once activated, caspases activate each other and co-operate in an intra-cellular cascade that results in the release of cytochrome C from the mitochondria and cell death (Thomson, 2001; Sjostrom & Bergh, 2001). Apoptosis is initiated by a wide range of external and internal sources including anticancer drugs, gamma and ultraviolet irradiation, deprivation of survival factors such as interleukin-1, and various cytokines that activate extra-cellular death-receptors such as the Fas and tumour necrosis factor receptors (Renehan *et al.*, 2001; Sjostrom & Bergh, 2001).

The bcl-2 family of genes contains at least 20 members which are either pro- or anti-apoptotic. Pro-apoptotic members include Bax, Bak, Bcl-x_S, BNIP3 and Bnip3L; anti-apoptotic members include Bcl-2, Bcl-x_L and survivin (Thomson, 2001). Survivin is a bifunctional member of the inhibitor of apoptosis gene family that counteracts cell death and controls mitotic progression. Survivin is present during embryonic development but is undetectable in most healthy human tissues (Kim *et al.*, 2003).

Butyrate treatment to cultured cells brings about changes in the expression of many apoptotic genes, some of which are discussed below. See table 1.4 for a summary of the genes whose expression is modulated by butyrate. Survivin expression in the normal human colon is localised to the crypt base, decreasing up the crypt and is inversely proportional to APC expression (Zhang *et al.*, 2001). The expression of survivin in HT-29 cells is markedly reduced upon treatment with butyrate (Daly *et al.*, 2005). HT-29 butyrate treated cells show a marked over expression of FAST, a serine/threonine

kinase that is rapidly activated during Fas-mediated apoptosis, compared to untreated cells (Iacomino *et al.*, 2001). Caspase-1 expression is enhanced in Caco-2 cells after treatment with NaBut. Caspase-3 is also up-regulated in Caco-2 cells as well as a RSB cell line (the latter obtained from a colonic tumour of an ulcerative colitis patient), although butyrate modulation of caspase-3 in RSB cells is to a lesser extent. Bak is strongly up-regulated in butyrate treated Caco-2 cells, while Bcl-2 is down-regulated, and Bak is up-regulated in butyrate treated RSB cells without an accompanying change in Bcl-2 (vivi-Green *et al.*, 2002). Bcl-2 was shown to be negatively modulated by butyrate when primary invasive human colonocytes were treated *in vitro* with SCFAs (Emenaker *et al.*, 2001). The expression of Bcl-2, Bcl-x_L, Bcl-x_S and Bak was found to decrease in Caco-2 cells after treatment with NaBut. The authors used a ribonuclease protection assay to identify apoptotic mRNA transcript expression but no protein expression was determined (Litvak *et al.*, 1998). Bcl-x_L has been shown to be negatively modulated by butyrate and Bak positively modulated by butyrate in HT-29, HCT-116 and AA/C1 cells; butyrate induced apoptosis in these three cell lines was demonstrated using a TUNEL assay (Cuff *et al.*, 2005). Modulation of Bcl-2 and Bak has also be shown in butyrate induced apoptosis in AA/C1, RG/C2 and PC/JW/F1, colonic adenoma cell lines (Hague & Paraskeva, 1995). Butyrate induced apoptosis in these cell lines was found to be p53-independent (Hague *et al.*, 1993) and overexpression of Bcl-2 did not protect against Bak-mediated apoptosis (Hague *et al.*, 1997). Furthermore, over-expression of Bcl-2 results in suppression of butyrate-induced apoptosis in DiFi, SW-480 and HCT-116 human colorectal carcinoma cell lines (Mandal *et al.*, 1997).

Treatment of bile acids has been shown to have a protective effect over butyrate-induced apoptosis. Treatment of secondary bile acids deoxycholic

acid and ursodeoxycholic acid markedly inhibited butyrate-induced apoptosis in AA/C1 cells, however no effect was shown in HT-29 cells. Secondary bile acids are known colonic tumour promoters and here the authors used concentrations of secondary bile acids that are typical of those found in the human colon (McMillan *et al.*, 2000). In summary, butyrate appears to play an important role in regulating proliferation, differentiation and apoptosis of colonic epithelial cells.

1.6.2.4 Other SCFAs

Treatment of propionate and valerate causes growth inhibition in HT-29, CaCo-2 and SW-1116 colonic epithelial cells and induced IAP and DPP IV activity; however this is to a lesser extent than butyrate treatment of the same concentration (Siavoshian *et al.*, 1997). Propionate and valerate induce IAP in HT-29 to a similar extent of butyrate. Propionate down-regulates CB1 but not to the same extent as butyrate, and valerate also down-regulates CB1 to a similar extent as butyrate (Hinnebusch *et al.*, 2002). The SCFAs propionate and acetate have been shown to induce apoptosis in three colonic adenoma cell lines (AA/C1, RG/C2 and PC/JW/F1). Propionate induced apoptosis to a lesser extent than butyrate with acetate even less, requiring 40 mM to induce apoptosis (Hague & Paraskeva, 1995).

1.7 Colorectal cancer

Colorectal cancer (CRC) has affected mankind for millennia; the earliest discoveries of CRC have been found in Egyptian and Peruvian mummies dating back to 3000 B.C. CRC, after lung (in men) and breast (in women) cancer, is the third most common cause of cancer related death in the world,

representing the second most common cause of cancer in the US (Rupnarain *et al.*, 2004). There are 30,000 new cases of CRC diagnosed in the UK every year which account for approximately 17,000 deaths per annum (Ilyas *et al.*, 1999). CRC is predominantly a disease of the Western world with the highest incidence rates found in North America, Australia and New Zealand. Africans, Asians and Latin Americans have the lowest rates of incidence and Western Europeans show higher rates of incidence over their Eastern counterparts (Ilyas *et al.*, 1999; Rupnarain *et al.*, 1991). CRC is influenced by diet and age and genetic predisposition (Rupnarain *et al.*, 2004); World wide research is now centred on elucidating the mechanisms involved in the activation and development of the disease in man. The various forms of CRC, its epidemiology and the aberrant gene-expression associated with the disease are all discussed below.

1.7.1 The adenoma to carcinoma sequence

The progression of a tumour is referred to as the adenoma to carcinoma sequence and outlines the progression of a colorectal cancer from normal mucosa through benign polyps, early and advanced adenoma to an invasive and metastatic adenocarcinoma (Renehan *et al.*, 2002). Tumour progression can be characterised with the development of qualitatively different stages, in a stepwise fashion, with each step involving one or multiple genetic alterations (Nowell, 2002). In total there are seven distinct events that characterise the progression from adenoma to carcinoma that are illustrated in figure 1.7. The latent interval for medium sized adenomas to develop malignancy is 5 to 7 years. Many small tumours (0.5 – 1 cm diameter) contain benign adenomas while large tumours are totally carcinomas (Eastwood, 1991). Aberrant crypt foci (ACF) are involved in the earliest steps in colorectal carcinogenesis. The

aberrant crypts appear larger, thicker and darker than normal and are found in clusters within the mucosa. ACF are divided into two types. Hypercellular ACF are the more common and have K-*ras* mutations, but are unlikely to progress to neoplastic lesions. Dysplastic ACF contain mutations of the adenomatosis polyposis coli (APC) gene and are precursors to adenomas (Renehan *et al.*, 2002). Polyps are outgrowths from the mucosa into the luminal space. Not all polyps progress to carcinoma or are considered neoplastic as some retain a benign status. Adenomatous and villous polyps are considered neoplastic and are commonly believed to be precursors to large intestine carcinomas (Shamsuddin, 1990). The development of polyps to carcinomas is correlated with inactivation of chromosome 18-associated, deleted-in-colorectal-cancer tumour suppressor gene (DCC). Inactivation/mutation of chromosome 17 p53 suppressor gene and the *ras* genes follow. The order of gene inactivation is always followed albeit not precisely (Rupnarain *et al.*, 2004; Nowell, 2002).

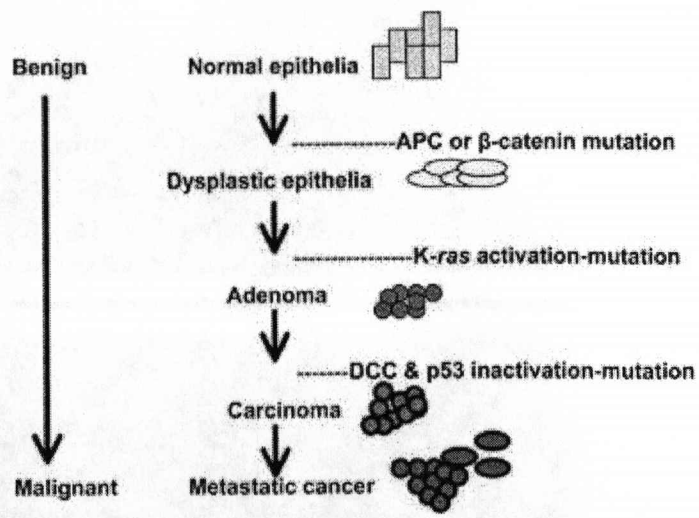


Figure 1.7 Illustration of the adenoma to carcinoma sequence

Adapted from Rupnarain *et al.*, (2004).

1.7.2 Familial CRC

Hereditary colorectal cancers are indicative of germ-line mutations that result in hereditary predispositions to cancer (Vogelstein & Kinzler, 2004). 20 % of CRC patients have a first or second degree relative with the disease, with hereditary patients making up 5 – 10 % of all CRC patients (Lynch & de la, 2003). There are two major forms; familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

FAP is characterised by a mutation in one APC allele predisposing FAP patients to CRC. FAP patients only require inactivation of the wt-APC allele to initiate tumourigenesis (Vogelstein & Kinzler, 2004), bringing early onset of cancer at around 20 – 30 years. APC controls the β-catenin-TCF pathway, a complex pathway that modulates cell proliferation and may play a role in the modulation of differentiation (Augenlicht *et al.*, 2002). The colon of FAP patients is littered with polyps that are adenomatous in type, with the number of polyps present, ranging from a few, to hundreds and thousands. In extreme cases the mucosa is not visible due to the numbers of polyps present (Shamsuddin, 1990). FAP patients have an almost 100 % risk of developing CRC, as the risk of developing cancer increases with the number of adenomas present (Eastwood, 1991). For each carcinoma present there may be up to a thousand benign adenomatous polyps (Knudson, 2002).

HNPCC patients have early onset of the disease which averages approximately 45 years. The HNPCC syndrome is an autosomal dominant condition that accounts for approximately 5 % of all colorectal cancers in the Western world (Ilyas *et al.*, 1999). HNPCC develops through germ-line mutations in any of the mismatch repair genes, which encode gene-products responsible for the repair of DNA base pair mismatches formed during DNA replication (Ilyas *et al.*, 1999; Knudson, 2002). The mismatch repair genes

MLH1 and MSH2 account for 90 % of HNPCC tumours, with a predominance for the disease in the right side of the colon (Lynch & de la Chapelle, 2003)

1.7.3 Sporadic CRC

Sporadic colorectal cancers represent the majority of colorectal tumours and are associated with late onset, usually 1 tumour over 6 to 7 decades of a life time. The onset of sporadic colorectal tumours requires the loss of both APC alleles, a factor adding to the increased onset compared to familial CRC (Augenlicht *et al.*, 2002). Sporadic CRC is characterised by mutations in a single somatic cell, usually epithelial in origin, resulting in the progression to a malignant and invasive tumour (Vogelstein & Kinzler, 2004).

1.7.4 Colorectal tumourigenesis and associated aberrant gene expression

The balance between proliferation and apoptosis is critical for the steady-state production of cell populations within the colon and in maintaining colonic tissue homeostasis. When rates of colonic cell proliferation increase and cell death is attenuated or inactivated, the risk of developing cancer is increased. In general deregulations of this mechanism can disrupt homeostasis, resulting in clonal expansion, with the resultant overproduction of affected cells (Lupton, 1995).

The human APC gene is localised to chromosome 5q21 in FAP patients. A mutation cluster region (MCR) has been identified in exon 15 of the *APC* gene in both familial and sporadic tumours, between codons 1286 and 1513. The MCR comprises only 10 % of the *APC* gene yet 90 % of all familial and sporadic *APC* mutations are found in this region (Smith *et al.*, 2002; Ilyas *et al.*, 1999). The majority of APC mutations are loss of function truncating mutations,

which result in the loss of APC activity and subsequent failure to modulate the β-catenin-TCF4 pathway (Ilyas *et al.*, 1999). Wt-APC transfected into HT-29 cells inhibits cell growth through increased apoptosis (Morin *et al.*, 1996).

Alterations in the machinery that controls whether the cell exits the resting phase and re-enter the cell cycle (G_0/G_1 phase), or progress from G_1 into S phase are found in virtually all tumour cells (Deshpande *et al.*, 2005). P53 acts as a suppressor of transformation in conjunction with *ras* oncogene (Finlay *et al.*, 1989). Functional inactivation of p53 by protein degradation or mutations within the gene are found in 70 % of CRC cases resulting in deregulation of p53-dependent apoptosis (Smith *et al.*, 2000; Sherr, 1996), aneuploidy and loss of p53 (Taylor *et al.*, 1999); p53 mutations are found to occur late in the adenoma-carcinoma sequence, (Kim *et al.*, 2001). Furthermore, loss of p53 predisposes cells to drug-induced gene amplification and aberrant chromosomal segregation during mitosis (Sherr, 1996). Mutations in K-ras are found in the majority of colorectal tumours. 50 % of colorectal tumours contain mutations in a *ras* gene, with 40 % of colorectal tumours containing a ki-*ras* mutation (Augenlicht, 1989).

Loss of pRb or hyperactivation of cdk4 and/or cdk6 are found in most human tumour cells. Hyperactivation can be achieved through loss or deregulation of CD expression, p16 or other INK4 family members (Deshpande *et al.*, 2005). The expression of p16 is disrupted in many cancers. $INK4a^{-/-}$ null mice spontaneously develop a multitude of cancers at six months old (Sherr, 1996). The proto-oncogene c-myc is deregulated in a wide variety of cancers and has been found to down-regulate p15, p21 and p27, with p21 being a major target. The regulation involves increased β-catenin-TCF4 activity and is correlated with increase c-*myc* expression (Gartel & Radhakrishnan, 2005). The anti-apoptotic gene *BIRC5* (survivin) is re-expressed in most human carcinomas.

Furthermore, survivin expression increases gradually from normal colorectal mucosa to adenomas with low grade dysplasia, adenomas with high grade dysplasia to carcinomas (Lin *et al.*, 2003).

CD1 is overexpressed in many cancers as a result of gene amplification or translocations targeting the CD1 locus, on human chromosome 11q13. Moreover, aberrant over expression of CD1 is seen in many CRC tumours (Sherr, 1996). Furthermore, approximately 50 % of HNPCC tumours exhibit overexpression of CD1. An early onset of HNPCC correlates with a polymorphism in CD1. The polymorphism (G870A) encodes an alternative splicing site that encodes a CD1 protein, which does not contain sequences involved in the turnover of the protein (Diehl, 2002). Investigations into the polymorphism found that HNPCC patients who were homozygous or heterozygous for the mutant allele developed colorectal cancer on average 11 years earlier (Kong *et al.*, 2000).

Table 1.5 some of the de-regulated genes during CRC development

Up-regulated in CRC	Down-regulated in CRC
Survivin	p53
c-myc	pRb
CD1	p15
K-ras	p16
Ki-ras	p21
β-catenin-TCF4	p27

1.7.5 Epidemiology of CRC and dietary fibre

The importance of dietary fibre was first identified by Burkitt in the 1970s. Burkitt observed a correlation between the high-fibre, low-fat diets, and low CRC incident rates in black Africans, compared to their white counterparts on

low-fibre, high-fat diets (Burkitt, 1971). Since the first observations by Burkitt into dietary fibre and CRC, there have been a great number of epidemiological studies performed investigating the link between high-fibre, low-fat diets and reduced CRC risks. Some cohort and human-intervention studies have found only a 50 % protective effect against CRC or no effect at all (Lipkin *et al.*, 1999; Topping & Clifton, 2001). A prospective study involving 88,757 women with a 16 year follow up period found no correlation between intake of dietary fibre and risk of CRC, when the first 6 follow up years were excluded (Fuchs *et al.*, 1999).

In general case-controlled studies have provided the most convincing results. In an analysis of 19 case-controlled studies, 13 showed a protective effect of dietary fibre against CRC. Fibre from cereals and pulses had the highest correlation (Lipkin *et al.*, 1999). An analysis of 13 case-controlled studies identified 12 which showed a decreased risk of CRC with increased dietary fibre intake. On average there was a 26 % reduced risk of CRC per 27 g of dietary fibre consumed per day (Howe *et al.*, 1992). A population based study involving 1993 CRC patients and 2410 controls in three areas of the U.S.A., showed a correlation between increased dietary fibre intake and reduced CRC risk. The study also found that increase fat intake from pre-prepared food also increased the risk of CRC. Furthermore, women with family histories of CRC had an increased risk of developing CRC compared to women on similar diets with no family history of CRC (Slattery *et al.*, 1997). A randomised-controlled study performed in the U.S.A., screened patients on the prostate, lung, colorectal and ovarian cancer screening study (PLCO study). In total 33971 patients were screened with 3591 adenomas discovered in the distal bowel (descending and sigmoid colon and rectum). The study identified a correlation between a high fibre diet and low colorectal adenomas. Furthermore the

correlation was highest with fibres from fruits and grains (Peters *et al.*, 2003). An EPIC cohort-study involving 519,978 people across 10 European countries (Denmark, France, Italy, Germany, Greece, Netherlands, Norway, Spain, Sweden and UK) showed an inverse correlation between total fibre intake and reduced CRC risk. The correlation was strong for the left side of the colon, but could not identify which fibres had the highest protective nature (Bingham *et al.*, 2003). A case control study performed in North Carolina, USA, found a diet high in fat and salt gave an increased risk of adenomas, whereas a diet high in fibre and fruit reduced the risk of adenomas. The risk of adenomas was also correlated with high secondary bile acids (Sandler *et al.*, 1993). A population (cohort) study in Japan observed CRC mortality rate decrease with increased rice and wheat consumption. However, a study in Hawaian Japanese observed no significant differences between colonic adenoma incident rate and intake of dietary carbohydrate (Young-in Kim, 2000).

A link between starch intake and reduced CRC risk has also been identified. High starch intake and reduced CRC risk has been observed in Africa and Australia, which have the same fibre intake but very different CRC incidence rates. Australians have higher CRC incidence rates compared to Africans but lower starch intake (Topping & Clifton, 2001). In general, incidences of CRC are inversely related to stool weight (Kirtchevsky, 1995), and a correlation exists between increased stool weight and increased intake of dietary fibre (Eastwood, 1991). However, the relationship between dietary fibre and cancer is a complex one. For example, faecal bile acid concentrations are higher in cancer patients (Bingham *et al.*, 2003), and the addition of bran to the diet has been found to reduce bile acid concentrations in the faeces, whereas pectin has been found to increase faecal bile acid concentrations (Eastwood, 1991). CRC patients tend to exhibit high faecal pH. Moreover, a study into CRC

incidences in African subpopulations found that faecal pH was highest in subpopulations with the highest CRC incidences rates. Intriguingly, the subpopulations all had approximately the same total fibre intake (Kritchevsky, 1995). Dietary fibre is not the only nutritional substance with implications in colon carcinogenesis; other factors are also important and include fat, bile acids, cholesterol plus minerals such as calcium (Bugaut & Bentejac, 1993; Eastwood, 1991).

Cancer is a multistage process that is likely to be affected at different stages by different dietary patterns, but as the discussion above demonstrates, the inclusion of high amounts of fibre in the diet does, in general, have a protective effect again colon carcinogenesis (Hill, 1995b).

1.8 Mechanistic action of butyrate

1.8.1 Intracellular Mechanism of butyrate action

Butyrate treatment of cultured cells results in modulation of multiple genes. The exact down stream processes by which butyrate modulates the vast number of genes is unclear; however some proposed methods through which butyrate's effects are mediated are discussed below.

Histones are acetylated by histone transacetylases on lysine residues and deacetylated by histone deacetylase. There is a correlation between the extent of histone acetylation and template activity of chromatin (Kruh *et al.*, 1994). Hyperacetylation of histones H3 and H4 leads to changes in chromatin structure; unfolding the chromatin and allowing access to the DNA by transcription factors and DNases (Hague & Paraskeva, 1995). Treatment of butyrate to cultured cells results in general histone acetylation through the inhibition of nuclear histone deactylases resulting in increase gene expression

(Clausen, 1995; Hague & Paraskeva, 1995). Increased DNA cleavage and micrococcal nuclease has been found to digest chromatin from butyrate treated cells *in vitro* to a much higher extent than untreated cells (Kruh *et al.*, 1994). Tricstatin A (TSA) is a known histone deacetylase inhibitor; treatment of cultured cells with TSA at concentrations that are known to inhibit histone deacetylase, has no effect on expression of p21. Moreover induction of IAP and DPP IV by TSA was significantly lower than that modulated by butyrate indicating that butyrate modulates p21 by some other means (Siavoshian *et al.*, 2000). Hyperacetylation of histones explains the global changes in gene expression seen in butyrate-treated cultured cells. Butyrate though, is able to modulate genes with a more discrete effect; hyperacetylation of histones does not explain this (Kruh *et al.*, 1995).

A family of nonhistone nuclear proteins known as the high mobility group (HMG) proteins have 3 subfamilies. One such subfamily, namely HMG-N1/N2 bind a 146 bp nucleosome core particle. Binding of HMG-N1/N2 to nucleosomes unfolds the higher order chromatin structure and enhances various DNA-dependent activities such as transcription and replication. Acetylation of HMG-N2 reduces its binding activity to chromatin. Butyrate treatment of HT-29 cells increased acetylation on HMG-N2 (Hinnebusch *et al.*, 2002) linking butyrate modulation of gene expression to unfolding of chromatin and increased gene expression.

Histone methylation and phosphorylation is also linked to modulation of chromatin structure. Histones methylation is inhibited in a concentration-dependent manner by butyrate. Butyrate has also been shown to inhibit the phosphorylation of histones H1 and H2A in HeLa cells (Boffa *et al.,* 1981). Moreover there is evidence indicating that histone H1 phosphorylation is required for G1/s progression (Boffa *et al.*, 1981; Clausen, 1995) and that

butyrate modulated inhibition of histone H1 phosphorylation and increased H3 phosphorylation is mediated by a calcium-dependent protein kinase (Kruh *et al.*, 1994).

Methylation of cytosine in the regulatory sequence of a gene prevents the expression of a gene. Treatment with butyrate has been shown to decrease DNA methylation (Clausen, 1995). Moreover, butyrate's effect on DNA methylation, whether to increase or decrease methylation, in dependent on cell type (Kruh *et al.*, 1994), illustrating another route by which butyrate's modulation of gene expression is mediated within the cell.

1.8.2 Mechanism of butyrate action at the plasma membrane

The mechanisms of butyrate regulation of gene expression discussed in section 1.8.1 are dependent on the presence of butyrate within the cell. There is evidence however, that butyrate is able to modulate the expression of certain genes extracellularly (Cuff *et al.*, 2005). As discussed previously, MCT1 is responsible for the transport of butyrate across the luminal membrane of colonocytes. Cuff *et al.*, (2005) knocked down the expression of MCT1 in three colonic cell lines (AA/C1, HT-29 and HCT-116) to investigate the effect on butyrate-induced gene expression on a variety of genes. After treatment of the colonic cell lines with small interfering RNA (siRNA) to MCT1, a decrease in MCT1 protein expression of up to 80 % was seen. A corresponding decrease in butyrate transport into colonic LMVs isolated from the MCT1 siRNA treated cells was also observed (Cuff *et al.*, 2005). Silencing MCT1 expression prevented butyrate-induced regulation of p21, IAP and CD1, in all three cell lines. However, the butyrate-induced modulation of the apoptotic genes *bak1* and *bcl-x*$_L$ was not inhibited by the knock-down of MCT1 (Cuff *et al.*, 2005). This finding has led to the hypothesis of the presence of a butyrate sensor

located on the luminal membrane of colonocytes. The putative butyrate-sensor senses butyrate in the lumen resulting in the downstream regulation of *bak1* and *bcl-x_L* (Cuff *et al.*, 2005).

1.9 Nutrient sensing

1.9.1 Nutrient sensing and regulation of gene expression

The intestinal lumen is a constantly changing environment responsive to the diet. The intestine is at constant equilibrium between the nutrients available in the lumen and the extra- and intra-cellular metabolic systems that are regulated by these nutrients. Nutrient-induced signalling pathways allow for optimal nutrient consumption through adaptations in metabolism and the associated adjustments in growth properties (Swinnen *et al.*, 2006). Polyunsaturated fatty acids (PUFA) bind intra-cellularly to members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors. Binding of PUFAs to PPARα induces up-regulation in β-oxidation enzymes (Al-Hasani & Joost, 2005). The intestine is also capable of sensing nutrients available in the lumen. Long-chain fatty acids are sensed (Raybould, 1999) and amino acid transporters are regulated via extra-cellular sensors (Hyde *et al.*, 2003). Glucose and galactose are sensed in the small intestine via a G-protein coupled receptor (GPCR) linked to the α-subunit of a G-protein, Gustducin, and a cAMP-PKA pathway (Dyer *et al.*, 2003); the activation of the sensor results in the downstream enhancement in expression of the intestinal sodium/glucose co-transporter, SGLT1 (Dyer *et al.*, 2003). The intestinal glucose sensor is thought to be a heterodimer of GPCRs T1R2 and T1R3 that are found in the taste buds on the tongue and the palate, sensing monosaccharides and artificial sweeteners. Furthermore, T1R2 and T1R3

have been found to be expressed along the axis of the mouse small intestine and in STC-1 cells, a mouse enteroendocrine cell line (Dyer *et al.*, 2005). The bitter taste receptors of the TR2 family have also been found to be expressed in mouse small intestine and STC-1 cells (Wu *et al.*, 2002). An extra-cellular calcium-sensing receptor (CaR) has been identified in the gastrointestinal tract (Hebert *et al.*, 2004). Upon activation, the receptor initiates intracellular pathways involving phophoinositide turnover, increased concentration of intra-cellular calcium and production of inositol trisphosphate (Riccardi & Maldonado-Perez, 2005). CaR is a member of the GPCR superfamily and is expressed along the entire length of the gastrointestinal tract (Kirchhoff & Geibel, 2006). CaR is not only activated by Ca^{2+} but by other divalent cations including Zn^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+} and Pb^{2+}; L-amino acids and small peptides; as well as the polyamines spermine and spermidine (Riccardi & Maldonado-Perez, 2005). Furthermore, CaR is expressed in the human large intestine on the apical and basolateral membranes of crypts as well as certain enteroendocrine cells (Hebert *et al.*, 2004). Colonic CaR regulates fluid secretion in colonic crypts through cAMP and cGMP pathways (Geibel *et al.*, 2006).

1.9.2 The SCFA receptors GRP41 and GPR43

Investigations carried out during the human genome project identified many orphan G-protein coupled receptors (GPCR); so called due to the lack of knowledge concerning their activating ligand. Brown et al., (2003) discovered that the orphan GPCRs, GPR41 and GPR43, are activated by short chain carboxylic anions in a dose-dependent and dose-specific manner (Brown *et al.*, 2003). A carbon chain length of between one to six carbons is required for activation, and the presence of two or more carboxylic groups fails to activate

the receptors (Le Poul *et al.*, 2003). Short-chain fatty acids, acetate, propionate, butyrate and pentanoate have been found to stimulate leptin production in adipocytes through GPR41, although acetate was found to be less potent than propionate, butyrate or pentanoate (Xiong *et al.*, 2004). Furthermore, GPR41 has been shown to induce apoptosis via a p53/Bax pathway during reoxygenation after ischemic hypoxia in HT-29 cells (Kimura *et al.*, 2001). The activation of GPR41 and GPR43 by SCFAs makes them excellent candidates for the putative human colonic butyrate sensor.

1.8 Aims and objectives

1.8.1 Synopsis

Butyrate is transported across the colonic luminal membrane predominantly by the monocarboxylate transporter isoform 1, MCT1. Silencing of MCT1 expression has been shown to have a profound inhibitory effect on the ability of butyrate to regulate some key butyrate responsive genes controlling proliferation and differentiation of colonic epithelial cells.

In contrast, inhibition of MCT1 expression has had no effect on the ability of butyrate to modulate the expression of genes regulating apoptosis; implying that for some butyrate responsive genes there is a need for butyrate entry into the cell, and for some butyrate may exert its effect via a plasma membrane butyrate sensor (Cuff *et al.*, 2005).

1.8.2 Aims

The aims of this study were to (i) synthesise a membrane impermeable butyrate analogue (disodium $PEG_{600}\alpha,\omega$-di(4-butyrate)) (ii) assess the structure and purity of the compound (iii) investigate whether the expression of genes regulating apoptosis of colonic epithelial cells are responsive to the membrane impermeable butyrate analogue and (iv) examine the significance of response in terms of butyrate regulation of gene expression.

CHAPTER 2

Methods

2.1 Materials

2.1.1 Chemicals and radioisotopes

All chemicals and solvents were of the highest analytical grade and obtained from Sigma-Aldrich (Poole, Dorset, UK), Fisher Scientific (Leics., UK), B.D.H Chemicals Ltd (Poole, Dorset, UK) or BioRad Laboratories (Herts., UK) unless otherwise stated.

[α-^{32}P]dCTP (specific radioactivity 3000 Ci mmol^{-1}) was obtained from the Radiochemical Centre (Amersham, Bucks., UK) and used within 1 half-life of the stated activity date. Sodium [U-^{14}C]butyrate was obtained from Sigma-Aldrich with a specific radioactivity of 16 mCi mmol^{-1}.

2.1.2 Antibodies

Antibodies to MCT1 were raised in rabbits against a peptide (CQKDTEGGPKEEESPV) corresponding to the C-terminus region of human MCT1. Monoclonal antibodies to human villin and human β-actin were purchased from the Binding Site (UK) and Sigma-Aldrich respectively. Polyclonal antibodies to human alkaline phosphatase were purchased from Abcam (UK). Polyclonal antibodies to Rat glut II were a kind gift from Prof. Bernard Thorens (University of Lausanne, Switzerland). Monoclonal antibodies to human Bcl-x_L and polyclonal antibodies to human Bak were purchased from Santa Cruz Technology Inc (UK). Mouse monoclonal antibodies to human HMC I (HLA-1); goat anti-mouse and swine anti-rabbit secondary antibodies conjugated to horseradish-peroxidase were purchased from Dako Ltd (UK).

2.2 Chemical Synthesis

2.2.1 Chemicals

4-Bromobutyronitrile and Poly(ethylene glycol)$_{600}$ were purchased from Lancaster Synthesis, Morecambe, UK. 1,8-Diazabicyclo[5.4.0]undec-7-ene, Diethylaminoethyl (DEAE) Sephadex A50, Triethylamine (99.5 %) and 1,4-dioxane (anhydrous Sure-seal™) were all obtained from Sigma-Aldrich, UK. All general solvents used were of Analar grade or equivalent.

2.2.2 Equipment and buffers

2.2.2.1 NMR

Analytical ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker AMX400 (400 MHz) NMR spectrometer (Bruker BioSpin Limited, Coventry, UK). Samples were run in deuterated methanol (CD_4OD) against an internal standard of tetramethylsilane (TMS). All chemical shifts are reported in ppm.

2.2.2.2 Mass spectrometry

Mass spectra were obtained on a Micromass LCT mass spectrometer (Waters Ltd, Herts., UK) using electrospray ionisation (EI) and direct infusion syringe pump sampling. All samples were diluted in methanol.

2.2.2.3 IR spectroscopy

Samples were prepared in nujol and held between NaCl discs. IR spectra were obtained using a Perkin-Elmer Spectrum RX1 FT-IR spectrometer (PerkinElmer Life And Analytical Sciences, Inc. Wellesley, MA, USA).

2.2.2.4 Preparation of triethylamine bicarbonate

Triethylamine bicarbonate (TEAB) was prepared according to (Eccleston & Trentham, 1977). A 1 M solution of TEA in distilled H_2O was prepared and carbon dioxide bubbled through the TEA solution for approximately three hours. The reaction was stopped once an approximate pH 7.5 was reached.

2.2.3 Thin Layer Chromatography

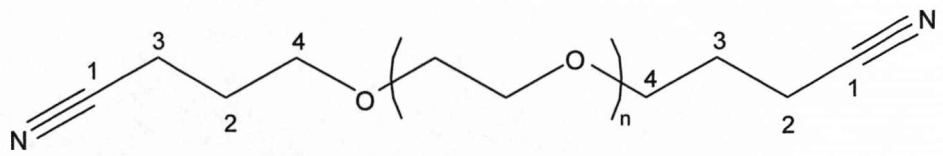
Aluminium-baked silica plates (Fisher, Leics., UK) were used, with an eluant consisting of ethyl acetate:methanol:27 % (w/w) concentrated ammonia:water (65:20:10:5 v/v/v/v).

The following stains were used:

- Iodine vapour; gave brown spots on a yellow background
- Potassium permanganate; gave yellow spots on a purple background
- Bromocresol green; gave blue spots on a pale blue/green yellow background

For use of the potassium permanganate and bromocresol green stains, the TLC plates were dipped into the corresponding stain and dried using a heat gun until spots were detected. When using iodine vapour, the TLC plate was left in a sealed container containing iodine for approximately 5 minutes.

2.2.4 The synthesis of di(4-butyronitrile)poly(ethylene glycol)$_{600}$

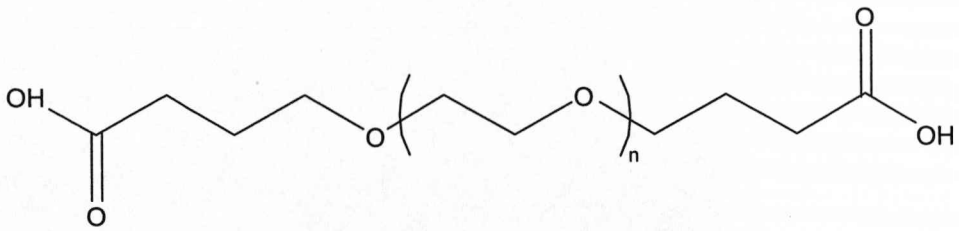


4-Bromobutyronitrile (BBN) (9.07 g, 61 mmol) was added to a stirred solution of poly(ethylene glycol) with an average molecular weight of 600 gmol^{-1} (6.0 g, 10 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (6.0 g, 39 mmol) in 10 ml dry 1,4-dioxane. The reaction mixture was heated at 110 °C at reflux for approximately 4 weeks until no starting material remained. The reaction was followed using TLC and an iodine vapour or potassium permanganate stains.

^{1}H NMR: δ_H (400 MHz, DMSO) 1.80 (PEG-BN, 4H, m, H2 and H3, $J_{3\text{-}4}$ = 6.2), 2.01 (BBN, 2H, m, H3, $J_{3\text{-}2}$ = 7.0 Hz, $J_{3\text{-}4}$ = 6.51 Hz), 2.13 (BBN, 2H, t, H1, $J_{2\text{-}3}$ = 7.0 Hz), 3.42 (PEG-BN, 2H, t, H4, $J_{4\text{-}3}$ = 5.88), 3.48 (BBN 2H, t, H4, $J_{4\text{-}3}$ = 6.50 Hz), 3.57 (PEG$_{600}$, 2H, t, H1′), 3.51 (PEG backbone, 4H, t, C$\underline{H_2}$-C$\underline{H_2}$-O). 3.57 (PEG$_{600}$, 2H, t, H2′, $J_{2'\text{-}1'}$ = 6.51)

Mass spectra: found (m/z) (4-butyronitrile)PEG$_{600}$ [M + Na]$^+$ n=10, 548.3; n=11, 592.3; n=12, 636.4; n=13, 680.4; n=14 724.4; n=15, 768.4; n=16, 812.5. di(4-butyronitrile)PEG$_{600}$ [M + Na]$^+$ n=9, 571.3; n=10, 615.3; n=11, 659.4; n=12, 703.4; n=13, 747.4; n=14, 791.4; n=15, 835.5. PEG$_{600}$ [M + Na]$^+$ n=7, 349.2; n=8, 393.2; n=9, 437.2; n=10, 481.3; n=11, 525.3; n=12, 569.3; n=13, 613.4; n=14, 657.5; n=15, 701.5.

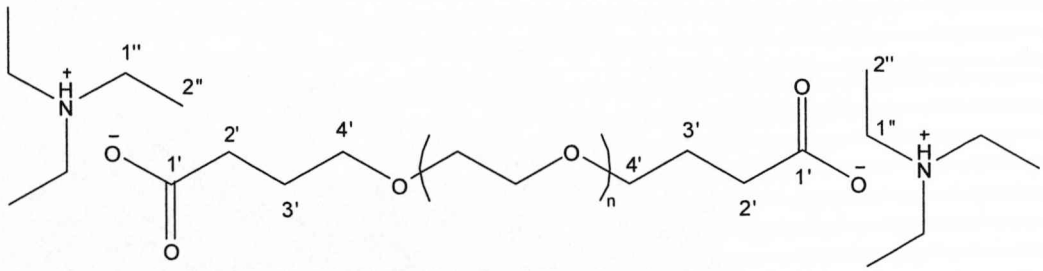
2.2.5 Hydrolysis of di(4-butyronitrile)poly(ethylene glycol)$_{600}$



The crude PEG_{600}butyronitrile was added to aqueous NaOH (20 ml 4 M) and stirred at 120 °C. The reaction was initially followed with TLC using iodine vapour, K_2MnO_4 and bromocresol green stains and then mass spectrometry. The reaction mixture was neutralised using 1 M HCl and concentrated prior to purification using anion-exchange column chromatography.

Mass spectra: found (m/z) (4-butyric acid)PEG_{600} $[M + Na]^+$ n=7, 435.2; n=8, 479.2; n=9, 523.2; n=10, 567.2; n=11 611.3; n=12, 655.3; n=13, 699.3; n=14, 743.2; n=15, 787.4; n=16, 831.4. di(4-butyic acid)PEG_{600} $[M + Na]^+$ n=6, 477.2; n=7, 521.2; n=8, 565.2; n=9, 609.2; n=10, 653.3; n=11, 697.3; n=12, 742.3; n=13, 785.3; n=14, 824.4; n=15, 868.4. (4-butyronitrile,4′-butyric acid)PEG_{600} $[M + Na]^+$ n=8, 546.2; n=9, 590.3; n=10, 634.3; n=11, 678.3; n=12, 722.3; n=13, 766.3.

2.2.6 Anion-exchange chromatography



Diethylaminoethyl (DEAE) Sephadex A50 (Sigma-Aldrich, UK) was used as the anion exchange resin. The concentrated reaction mixture (5 ml, ~5g) was added onto the column with dH_2O. The column was washed using dH_2O, to remove unreactive starting material and eluted using 1 M triethylamine bicarbonate (TEAB). The fractions containing $PEG_{600}\alpha,\omega$-di(4-butyric acid), as determined using TLC, were pooled and the solvent removed by rotary evaporation, to yield a light brown oil.

^1H NMR: δ_H (400 MHz, CD_3OD) 1.28 (TEAH, 3H, t, H2″, $J_{2''\text{-}1''}$ = 7.31 Hz), 1.67 (PEG-BN, 4H, m, H2 and H3, J3-4 = 7.47), 1.86 (PEG-ba, 2H, m, H3′, $J_{3'\text{-}2'}$ = 6.83 Hz, $J_{3'\text{-}4'}$ = 7.47 Hz), 2.21 (PEG-ba, 2H, t, H2′, $J_{2'\text{-}3'}$ = 7.29 Hz), 3.25 (TEAH, 2H, q, H2″, $J_{2''\text{-}3''}$ = 7.15 Hz), 3.46 (PEG-BN, 2H, t, H4, $J_{4\text{-}3}$ = 7.15 Hz), 3.51 (PEG-ba, 2H, t, H3′, $J_{4'\text{-}3'}$ = 6.52 Hz), 3.67 (PEG backbone, 4H, t, $C\underline{H_2}$-$C\underline{H_2}$-O).

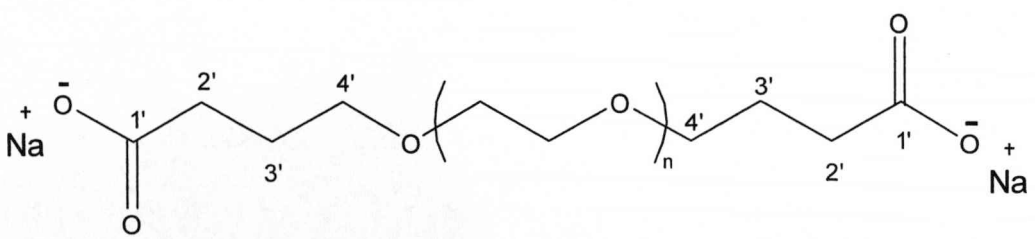
Mass spectra: found (m/z) (4-butyric acid)PEG_{600} $[M + Na]^+$ n=7, 435.2; n=8, 479.2; n=9, 523.2; n=10, 567.2; n=11 611.3; n=12, 655.3; n=13, 699.3; n=14, 743.2; n=15, 787.4; n=16, 831.4. di(4-butyic acid)PEG_{600} $[M + Na]^+$ n=6, 477.2; n=7, 521.2; n=8, 565.2; n=9, 609.2; n=10, 653.3; n=11, 697.3; n=12, 742.3; n=13, 785.3; n=14, 824.4; n=15, 868.4. (4-butyronitrile,4′-butyric acid)PEG_{600}

$[M + Na]^+$ n=8, 546.2; n=9, 590.3; n=10, 634.3; n=11, 678.3; n=12, 722.3; n=13, 766.3.

2.2.6.1 Extraction of di(4-butyric acid)poly(ethylene glycol)$_{600}$

A portion of the crude product (approximately 50 g) from the anion exchange chromatography was stirred for 48 hours in a large excess of acetone (400 ml). The acetone was filtered to remove the precipitated TEAH and the filtrate evaporated to yield a light brown oil. Another volume of acetone was added to precipitate more TEAH salt before filtering and evaporating the filtrate. The washing and filtering was repeated several times to reduce the amount of TEAH to a minimum. The amount of TEAH present was constantly monitored using ^{1}H NMR.

2.2.7 Sodium-exchange chromatography



Cellex-P, hydrogen ion form (BioRad, UK) was mixed with aqueous NaOH (0.5 M, ~ 25 ml) and stirred at room temperature for 15 minutes to produce the sodium ion form of the resin. The Cellex-P sodium form (~ 30 ml) was poured into a column and cleaned by washing the column three times with dH_2O, which also removed excess sodium ions. The $PEG_{600}\alpha,\omega$-di(4-butyric acid) TEAH salt (~ 5 g) was dissolved into a small volume of dH_2O before loading

on to the column and eluted with dH_2O. The fractions containing di(4-butyric acid)PEG_{600}, as determined using TLC, were pooled and the solvent removed to yield a brown oil. The volume of deionised distilled water was added to prepare a 200 mM solution of disodium PEG_{600}di(4-butyrate) and aliquoted into 1 ml conical microcentrifuge tubes, afterwhich the deionised distilled water was removed in vacuo.

^1H NMR: δ_H (400 MHz, CD_3OD) 1.85 (PEG-ba, 2H, m, H3, $J_{3'-2'}$ = 7.4 Hz, $J_{2'-3'}$ = 6.83 Hz), 2.26 (PEG-ba, 2H, t, H2′, $J_{2'-3'}$ = 7.4 Hz), 3.48 (PEG-ba, 2H, t, H4′, $J_{4'-3'}$ = 6.45 Hz), 3.64 (PEG backbone, 4H, t, ($C\underline{H}_2$-$C\underline{H}_2$-O)$_n$).

^{13}C NMR: δ_C (400 MHz, CD_3OD) 18.8 (C2), 24.4 (C3′), 27.2 (C3), 32 (C2′), 71.3 (C<u>H</u>2-C<u>H</u>2-O-), 71.9 (C4′), 73.5 (C4), 180 (C1′).

Mass spectra: found (m/z) (4-butyric acid)PEG_{600} $[M + Na]^+$ n=7, 435.2; n=8, 479.2; n=9, 523.2; n=10, 567.2; n=11 611.3; n=12, 655.3; n=13, 699.3; n=14, 743.2; n=15, 787.4; n=16, 831.4. di(4-butyic acid)PEG_{600} $[M + Na]^+$ n=6, 477.2; n=7, 521.2; n=8, 565.2; n=9, 609.2; n=10, 653.3; n=11, 697.3; n=12, 742.3; n=13, 785.3; n=14, 824.4; n=15, 868.4. (4-butyronitrile,4′-butyric acidPEG_{600}) $[M + Na]^+$ n=8, 546.2; n=9, 590.3; n=10, 634.3; n=11, 678.3; n=12, 722.3; n=13, 766.3.

2.3 Tissue and cell culture

2.3.1 Tissue collection

Porcine colonic samples were taken from adults pigs at an abattoir. Sections of the intestine were removed, divided into proximal, middle and distal sections and rinsed in ice-cold saline (0.9 % (w/v) NaCl pH 7.4). The sections were cut open and blotted with paper towels to remove excess mucous. The mucosa was scraped off with a glass slide, wrapped in aluminium foil and immediately frozen in liquid nitrogen. Upon returning to the laboratory the mucosal scrapings were stored at –80 °C until use.

2.3.2 Routine maintenance of cell lines

AA/C1, a human colonic epithelial non-tumorigenic adenoma-derived cell line (Williams *et al.*, 1990), was a kind gift from Professor C. Paraskeva, University of Bristol, UK. AA/C1 cells were maintained at 37 °C with 5 % CO_2 in glutamine-free Dulbecco's modified eagle's medium (DEME; Sigma-Aldrich, UK), supplemented with, 20 % (v/v) foetal bovine serum (Sera Labs., UK), 100 $\mu g\ ml^{-1}$ streptomycin, 100 units ml^{-1} penicillin, 1 $\mu g\ ml^{-1}$ hydrocortisone sodium succinate, 0.2 units ml^{-1} human Actrapid insulin (Novo Nordisk, Switzerland) and 2 mM glutamine.

HT-29 cells, an adenocarcinoma cell line, were also a kind gift from Professor C. Paraskeva and were maintained at 37 °C with 5 % CO_2 in DEME, supplemented with 10 % (v/v) foetal bovine serum, 100 $\mu g\ ml^{-1}$ streptomycin, 100 units ml^{-1} penicillin and 2 mM glutamine.

Cells were routinely passaged using a solution of 0.1 % (w/v) tripsin, phosphate buffered saline (Sigma-Aldrich, UK) and 0.1 % (w/v) EDTA. AA/C1

cells were used between passage numbers 83 and 90; HT-29 cells were used between passage numbers 13 and 34.

2.3.3 Treatment of cultured cells

Cells were grown to either 70 % confluency (HT-29) or 100 % confluency (AA/C1) for treatment. Sodium butyrate (Sigma-Aldrich), sodium heptafluorobutyrate (Sigma-Aldrich), poly(ethylene glycol)$_{600}$ (Lancaster synthesis, UK) and disodium PEG$_{600}\alpha,\omega$-di(4-butyrate) were prepared using ddH_2O to a final concentration of 200 mM. Cells were treated with compounds (2-5 mM) for up to 48 hours and resupplemented after 24 hours. After the elapsed time of treatment, cells were washed three times with phosphate buffered saline (PBS) and harvested with a cell scraper. Cells were collected by centrifugation and either used immediately or stored at -80 °C until use.

2.4 Preparation of protein samples

2.4.1 Preparation of whole cellular homogenate from cultured cells

Cell pellets were resuspended in lysis buffer (10 mM Hepes/tris pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.1 % (w/v) SDS, 1 x Complete protease inhibitor (Boehringer Mannheim, Germany) and 0.2 mM PMSF), transferred to a 1.5 ml microfuge tube and mixed 10 times using a 1 ml pipette. The cells were homogenised using either a 27 G needle or a 100 µl Hamilton syringe (Scientific Glass Engineering, PTY, Ltd, Australia), depending on the volume of lysis buffer used, by drawing the cellular suspension through the needle 10 times. Whole cell homogenates were stored at -20 °C.

2.4.2 Preparation of porcine colonic luminal membrane vesicles

Mucosal scrapings were defrosted on ice in a hypotonic buffer, buffer 1 (100 mM mannitol, 2 mM Hepes/tris pH 7.1, 0.2 mM benzamidine, 0.2 mM PMSF, 0.2 mM DTT). Approximately 10 ml buffer per 1 g tissue was used. The scrapings were homogenised using an Ystral polytron (Ystral Scientific Instruments, Cambridge, UK) with a medium probe on setting 4 for 2 x 30 seconds with the sample kept on ice during the interval. The homogenate was filtered through 2 layers of gauze and made up to 100 ml total volume with buffer 1 before centrifuging at 500 x g for 10 minutes at 4 °C (SS34 rotor, Sorvall RC 5C Plus, Kendro Laboratory Products, USA) to remove nuclei. The supernatant (S1) was decanted and placed on ice. $MgCl_2$ was added to a final concentration of 10 mM and left to stir for 20 minutes before centrifuging at 3000 x g for 10 minutes at 4 °C (SS34 rotor) to remove organelles and solublised membranes. The supernatant (S2) was centrifuged at 39,000 x g for 30 minutes at 4 °C (SS34 rotor) to collect the luminal membranes. The pellet (P3) was resuspended in 6 ml buffer 2 (100 mM mannitol, 20 mM Hepes/tris pH 7.4, 0.1 mM $MgSO_4$) and homogenised using a Dounce homogeniser (Jencons Ltd, UK) with a tight fitting Teflon pestle for 60 strokes, before centrifuging at 39,000 x g for 45 minutes at 4 °C (SS34 rotor) to yield the purified luminal membranes. The pellet (P4) was resuspended in 50 µl buffer 3 (300 mM mannitol, 20 mM Hepes/tris pH 7.4, 0.1 mM $MgSO_4$) and homogenised using a 25 G needle. The colonic luminal membrane vesicles were either used on the day or stored under liquid N_2 until further use.

2.4.3 Quantification of protein: micro assay.

Protein concentrations were determined by the Bio-Rad assay technique based on the method of (Bradford, 1976), exploiting the shift in absorbance from 465 nm to 595 nm when proteins bind to Coomassie blue. A commercially available dye was used (BioRad, Herts, UK) and a standard curve prepared (3 μg to 24 μg) using porcine γ-globulin (1.5 $mgml^{-1}$) as the standard in 800 μl buffer 3. 1 μl of sample was diluted in 800 μl of buffer 3 (see section 2.4.2) and 200 μl of protein dye was added to all samples and standards before leaving for 15 minutes at room temperature. The absorbance of samples and standards was read at 595 nm on a UV spectrophotometer (Hitachi U-200 Spectrophotometer, UK).

2.5 Enzyme Assays

2.5.1 Measurement of cysteine-sensitive alkaline phosphatase activity

The activity of cysteine-sensitive alkaline phosphatase, a luminal membrane marker, was measured according to the procedure of Brasitus & Keresztes, (1984), with *p*-nitrophenylphosphate (PNPP) as the substrate.

Samples (50 μg) were incubated at 37 °C and the reaction started with the addition of 900 μl of reaction buffer (32 mM glycine pH 9.3, 3.2 mM $MgCl_2$, 0.32 mM $ZnSO_4$, 15 mM PNPP). The samples were incubated for 15 minutes in the presence or absence of 10 mM L-cysteine before stopping the reaction with the addition of 2 ml of 1 M NaOH. The absorbance was read at 410 nm (E_{mM} = 17) against a buffer blank. Specific activity was expressed as nmol. min^{-1} $(mg\ protein)^{-1}$. The appearance of *p*-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

2.5.2 Measurement of α-mannosidase activity

The activity of α-mannosidase, a marker for the golgi apparatus, was measured according to the procedure of Tulsiani *et al.*, (1977), with *p*-nitrophenol-α,D-mannoside (PNPM) as the substrate.

Colonic LMV fractions (50 μg) were incubated at 37 °C in 500 μl of a citrate buffer (50 mM citric acid, 50 mM sodium citrate, pH 4.5). The reaction was started with the addition of 500 μl of substrate (10 mM PNPM) and incubated for 30 minutes at 37 °C. The reaction was terminated with the addition of 2 ml of a borate buffer (0.2 mM boric acid pH 9.8 with 2 M NaOH) and the fractions were left for 15 minutes for the yellow colour to develop before the absorbance was read at 410 nm (E_{mM} = 18.5) against a buffer blank. Specific activity was expressed as nmol. min^{-1} (mg protein)$^{-1}$. The appearance of *p*-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

2.5.3 Measurement of Succinate dehdrogenase activity

The activity of succinate dehydrogenase, a marker of the mitochondria, was measured according to the procedure of Pennington, (1961) with *p*-iodotetrozolium succinate (INT) as the substrate.

A 2x Reaction buffer was prepared consiting of: 100 mM sodium succinate, 50 mM sucrose, 50 mM potassium dihydrogen phosphate (KH_2PO_4) and 50 mM dipotassium phosphate (K_2PO_4) pH 7.4

On the day of the experiment the 2x reaction buffer was diluted two fold and substrate was added to a final concentration of 1 mg/ml. Colonic LMV fractions (30-40 μg) were incubated at 37 °C in glass tubes; the reaction started with the addition of 750 μl of the reaction buffer and left to incubate until a rose colour

appeared. 3 ml of ethyl acetate was added to each sample and mixed to extract the tetrazolium dye product before reading the upper phase at 490 nm (E_{mM} = 20.1) against a buffer blank. The appearance of the tetrazolium product was shown to be linear with time and protein concentration under the parameters of the assay.

2.5.4 Measurement of tris-resistant-α-glucosidase activity

The activity of tris-resistant-α-glucosidase, a marker for the endoplasmic reticulum, was assayed according to Peters, (1976), with *p*-nitophenyl-α,D-glucoside (PNPG) as the substate. 50 µg of sample were incubated on ice in 50 µl of tris buffer (35 mM tris/HCl pH 8.0, 5 mM glutathione) for 15 minutes. The samples were then incubated at 37 °C and the reaction was started by the addition of 250 µl phosphate buffer (0.1 mM Na_2HPO_4, 0.21 mM *p*-nitophenyl-α,D-glucoside, 0.1 % (v/v) triton X-100). After 30 minutes the reaction was stopped by the addition of a glycine buffer (5 mM glycine pH 10.4) and the absorbance read at 410 nm (E_{mM} = 18.5) against a buffer blank. Specific activity was expressed as nmol. min^{-1} (mg protein)$^{-1}$. The appearance of *p*-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

2.5.5 Measurement of H^+/K^+, Na^+/K^+-ATPase activity

The activity of the H^+/K^+ ATPase was measured using an assay adapted from Sarkar (2002). All solutions were prepared fresh on the day of the assay. A 2 x assay mix consisting of 240 mM NaCl, 8 mM Na_2ATP, 8 mM $MgCl_2$, 120 mM tris/HCl pH 7.5 and 2 mM EDTA was prepared and diluted 2 fold to produce 2 reaction solutions:

Solution A (Total ATPase activity): 1 x assay mix with 20 mM KCl

Solution B (K^+ insensitive): 1 x assay mix

Protein samples (50 µl) were permeablised with saponin (0.05 % (w/v) final concentration) and incubated at 37 °C. The reaction was started with the addition of 400 µl of either solution A or B and left to incubate for 10 minutes. The reaction was stopped by the addition of 1 ml developing solution (1.15 M H_2SO_4, 1 % (w/v) ammonium molybdate, 4 % (w/v) ferrous sulphate) and left to develop for 1 hour. The developing solution has a light grey colour that changes to a deep blue during development. A standard curve of K_2HPO_4 was also prepared from 0 to 720 µM, to calculate the P_i release, which was developed at the same time as the samples. Buffer blanks were used for each solution (A and B) that consisted of 50 µl ddH_2O and 400 µl of the corresponding solution. The absorbance was measured at 690 nm and specific activity was expressed as the amount of P_i released in nmol. min^{-1} (mg protein)$^{-1}$. The activity of H^+/K^+-ATPase was determined by subtracting the specific activity of solution B from solution A.

2.5.6 Measurement of alkaline phosphatase activity.

The total activity of alkaline phosphatase was measured based on the method of Shirazi *et al.*, (1981) with PNPP as the substrate. A reaction buffer was prepared in advance consisting of: 10 mM sodium bicarbonate ($NaHCO_3$), 10 mM disodium carbonate (Na_2CO_3), 0.1 mM magnesium sulphate ($MgSO_4$).

On the day of the assay the reaction buffer was mixed 9:1 (v/v) with 10 mM PNPP. Samples were incubated at 37 °C along with the reaction buffer and the reaction started by the addition of 1 ml reaction buffer. The reaction preceded for 10 minutes at 37 °C and was terminated by the addition of 1 ml 0.5 M NaOH and the absorbance read at 410 nm (E_{mM} = 17) against a buffer blank. Specific activity was expressed as nmol. min^{-1} (mg protein)$^{-1}$.

2.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

2.6.1 Preparation of samples

Protein samples (10 – 30 μg) were diluted, usually 1:4 (v/v), with denaturing sample buffer (2 % (w/v) SDS, 62.5 mM tris/HCl pH 6.8, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol and 0.01 % (w/v) bromophenol blue) and heated in boiling water for 4 minutes. The samples were centrifuged briefly (Eppendorf bench top centrifuge 5415C, UK) before loading into the wells.

2.6.2 Separation of proteins on SDS-PAGE gels.

SDS-PAGE gels were prepared based on the method of (Laemmli, 1970). The Bio-Rad mini PROTEAN II apparatus was used (Bio-Rad, Herts., UK). 2 glass plates and 2 spacers (0.75 mm thick) were cleaned with ethanol and assembled with the spacers lining the outside edge of the plates. The Running gel was prepared according to table 2.1 and poured in between the glass plates to a distance of 2 cm from the top, bubble free. A thin layer of butanol-saturated water was added and the gel was left to polymerise for 30 minutes. After polymerisation the butanol-saturated water was removed and a plastic

comb inserted into the top of the plates. The stacking gel was prepared according to table 2.2 and poured onto the top of the running gel bubble free and left to polymerise for 30 minutes.

Table 2.1 Preparation of running gel

	8 %	10 %	12 %
ddH_2O (ml)	5.4	4.9	4.4
0.5 M tris/HCl pH 6.8 (ml)	2.5	2.5	2.5
40 % (v/v) acrylamide/BIS (ml)	2	2.5	3
20 % (w/v) SDS (ml)	0.05	0.05	0.05
TEMED (ml)	0.005	0.005	0.005
20 % (w/v) APS (ml)	0.05	0.05	0.05

The polymerised gels were placed into a buffer chamber containing 1.44 % (w/v) glycine, 0.3 % (w/v) tris base and 0.1 % (w/v) SDS. The gels were loaded with the protein samples and a molecular weight marker ladder and run at 11 mA/gel until the dye front had reached the bottom of the gel.

2.6.3 Electrotransfer of proteins to PVDF membrane.

Polyvinyldifluoride (PVDF) membrane (Imunno-Blot PVDF, BioRad, UK) was equilibrated in transfer buffer (0.15 M glycine, 20 mM Trizma base and 20 % (v/v) methanol). The polyacrylamide gels were carefully removed from the glass plates and the stacking gel discarded. The running gel was placed into transfer buffer before placing into the transfer cassette bottom up in the following order: sponge pad, foam pad, 3 MM filter paper, running gel, PVDF membrane, 3 MM filter paper, foam pad and sponge pad. The cassette was closed and squeezed to remove bubbles before placing into the transfer tank. The transfer tank was filled with transfer buffer and electrotransfer took place

at 100 V for 60 to 90 minutes depending on the acrylamide percentage of the running gel.

Table 2.2 Preparation of stacking gel

	4%
ddH_2O (ml)	6.4
0.5 M tris/HCl pH 6.8 (ml)	2.5
40 % (v/v) acrylamide/BIS (ml)	1
20 % (w/v) SDS (ml)	0.05
TEMED (ml)	0.01
20 % (w/v) APS (ml)	0.05

2.7 Detection of Proteins

2.7.1 Staining with Ponceau Red S.

In order to assess successful protein transfer the PVDF membranes were stained with Ponceau Red (1 % (w/v) Ponceau S Red and 3 % (w/v) TCA). The membranes were removed from the PROTEAN II apparatus and rinsed in ddH_2O before covering with Ponceau Red for 30 seconds to 1 minute. The membranes were washed carefully with a small volume of ddH_2O to remove background staining and to visualise the protein bands. The membranes were de-stained with sequential washes in ddH_2O. The clean membranes were trimmed and either used immediately or air dried, wrapped in paper towel and frozen at -20 °C until use.

2.7.2 Immuno-detection of MCT1 protein in porcine colonic luminal membrane vesicles

Non-specific proteins were blocked by incubating the membranes at room temperature in buffer consisting of PBS, 5 % (w/v) skimmed milk powder (Oxoid Ltd, UK), 0.05 % (v/v) tween-20 for 1 hour at room temperature. The membranes were washed with incubation buffer (PBS, 1 % (w/v) skimmed milk powder, 0.05 % (v/v) tween-20) for 10 minutes before incubating at room temperature in fresh incubation buffer; with the primary antibodies at a dilution of 1:5000. Following incubation the membranes were washed 3 times with incubation buffer. Incubation at room temperature with secondary antibodies, affinity purified swine anti-rabbit, at a dilution of 1:2000 then followed for 1 hour. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.3 Immuno-dection of MCT1 protein in cultured cells

Immuno-dection of MCT1 in cultured cells was carried out using the same protocol for the detection of MCT1 protein in colonic luminal membrane vesicles with the following exceptions: all incubations were carried out in TTBS pH 7.4 (0.05 % (v/v) Tween 20, 0.5 M NaCl, 10 mM Trizma base) with 5 % skimmed milk powder.

Non-specific proteins were blocked by incubating the membranes at room temperature in incubation buffer (TTBS pH 7.4 (0.05 % (v/v) Tween 20, 0.5 M NaCl, 10 mM Trizma base) with 5 % skimmed milk powder) at room temperature. The membranes were incubated at room temperature in fresh incubation buffer; with the primary antibodies at a dilution of 1:5000. Following incubation the membranes were washed 3 times with incubation buffer.

Incubation at room temperature with secondary antibodies, affinity purified swine anti-rabbit, at a dilution of 1:2000 then followed for 1 hour. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.4 Immuno-detection of Villin and β-Actin proteins

Non-specific proteins were blocked by incubating the membranes at room temperature in a buffer consisting of PBS, 5 % (w/v) skimmed milk powder, 0.1 mM EDTA and 0.5 % (v/v) triton X-100 for 1 hour. The membranes were washed in incubation buffer consisting of PBS, 1 % (w/v) skimmed milk powder, 0.1 mM EDTA and 0.5 % (v/v) triton X-100 for 10 minutes. The membranes were incubated in fresh incubation buffer for 1 hour at room temperature with the primary antibodies at a dilution of 1/2000, anti-villin; and 1:10000, anti-β-actin. Following incubation the membranes were washed 3 times with incubation buffer. Incubation at room temperature with secondary antibodies, affinity purified goat anti-mouse, then followed at a dilution of 1:2000 for 1 hour. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.5 Immuno-detection of HLA-1 protein

Non-specific proteins were blocked by incubating the membranes at room temperature in a buffer consisting of TTBS (500 mM NaCl, 10 mM Trizma base, 0.05 % (v/v) tween-20 pH 7.4) and 5 % (w/v) skimmed milk powder for 1 hour at room temperature. The membranes were washed in incubation buffer consisting of TTBS and 1 % (w/v) skimmed milk powder for 10 minutes. The membranes were incubated in fresh incubation buffer for 1 hour at room

temperature with the primary anti-HLA-1 antibodies at a dilution of 1:1000. Following incubation, the membranes were washed 3 times in incubation buffer. The membranes were incubated with the secondary antibodies, affinity purified goat anti-mouse, at a dilution of 1:2000 for 1 hour at room temperature. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.6 Immuno-detection of Bcl-x_L and Bak proteins

Non-specific proteins were blocked by incubating the membranes at room temperature in a buffer consisting of TBS-TM (100 mM NaCl, 10 mM Trizma base, 0.1 % (v/v) tween-20 pH 7.4, 5 % (w/v) skimmed milk powder) for 1 hour at room temperature. The membranes were incubated in fresh incubation buffer for 1 hour at room temperature with the respective primary antibodies at a dilution of 1:200. Following incubation, the membranes were washed 3 times in incubation buffer. The membranes were incubated with the secondary antibodies, affinity purified goat anti-mouse, bcl-x_L; swine anti-rabbit, bak, at a dilution of 1:2000 for 1 hour at room temperature. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.7 Immuno-detection of p21 protein

Non-specific proteins were blocked by incubating the membranes at room temperature in TBS-TM buffer for 1 hour at room temperature. The membranes were washed in incubation buffer (100 mM NaCl, 10 mM Trizma base, 0.1 % (v/v) tween-20 pH 7.4, 1 % (w/v) skimmed milk powder) for 10 minutes before incubated in fresh incubation buffer for 1 hour at room

temperature with primary antibodies at a dilution of 1:500. Following incubation, the membranes were washed 3 times in incubation buffer. The membranes were incubated with the secondary antibodies, affinity purified goat anti-mouse at a dilution of 1:2000 for 1 hour at room temperature. The membranes were then washed 3 times as above before developing the signal and exposing to film (section 2.7.6).

2.7.8 Development of immunoblots

The antigen-antibody binding was detected using the Enhanced Chemiluminescence (ECL) system (Amersham International, UK) following the manufacturers instructions. The membranes were incubated in developer solution (equal volume of solution 1 and solution 2) for 1 minute. Excess developer solution was removed by blotting the corner of the membrane on paper towel before wrapping the membrane in Saran Wrap. The membranes were exposed to BioMax-ML (Kodak, UK) film for 5 seconds – 15 minutes before developing the film. The bands present on the film were quantified using scanning densitometry computer software (Phoretix ID quantifier, Non-linear Dynamics).

2.7.9 Stripping and reprobing of PVDF membranes.

PVDF membranes were stripped of the primary and secondary antibodies in order to reprobe the membrane with a different primary antibody. Membranes were washed 3 times in an acidic stripping buffer (137 mM NaCl and 20 Mm glycine pH 2.5) for 15 minutes on a rocking platform; followed by a 10 minute wash in the buffer of the proceeding antibody. Following stripping of the

membranes, immunoblotting for a second protein was carried out as described previously.

2.8 Transport studies with butyrate into colonic LMVs

The transport of butyrate into colonic LMVs was carried out using the rapid filtration technique as described by (Shirazi *et al.*, 1981). On the day of the experiment, LMV were thawed on ice and 50 μg of protein (2.5-7 μl) per assay were aliquoted. The uptake was carried out at 37 °C; started with the addition of 100 μl of uptake buffer (see figure legends for exact buffer composition) and incubated for 5 s. The uptake was stopped with the addition of 1 ml of ice cold stop buffer (100 mM mannitol, 100 mM sodium gluconate, 20 mM Hepes/tris pH 7.5, 1 mM sodium butyrate). The solution was immediately mixed using a whirly mixer before 900 μl were removed and filtered through pre-wetted 0.2 μm filters (Millipore GSTF02500 Millipore, Herts., UK). The filter was washed five times using 1 ml of ice cold stop buffer to remove excess radio-labelled butyrate before placing into a scintillation vial with 4 ml scintillation cocktail (OptiPhase 'HiSafe' 2 Liquid Scintillation Cocktail, PerkinElmer, Leics, UK). The samples were counted in a liquid scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, CA, USA). Zero time blanks were performed to account for non-specific binding of the radio-labelled butyrate to the vesicles; here stop solution was added to the vesicles first, before the uptake buffer. The values obtained were subtracted from the corresponding 5 s uptake. An internal standard (50 μl of a random unfiltered stopped reaction mixture) was used to calculate the concentration of butyrate uptake. Butyrate uptake was measured in the presence of 1 mM sodium butyrate with tracer amounts of [U-^{14}C]-butyrate to give final counts of 30,000

cpm in 50 μl standards. Results of butyrate uptake are expressed as pmol. (mg protein)$^{-1}$ (unit time)$^{-1}$.

2.9 Isolation of total RNA

Total RNA was isolated using the Qiagen RNeasy Kit (Qiagen Ltd, Crawley, West Sussex, UK), following the manufacturers' instructions. Briefly: cell pellets, if frozen, were defrosted at room temperature for no more than five minutes before 600 μl of lysis buffer was added. The pellets were quickly resuspended in lysis buffer before removing to a 1.5 ml microfuge tube and mixed with a 10 ml pipette 10 times. The pellets were homogenised using a 25 G needle before 600 μl of 70 % (v/v) ethanol was added; the suspension was mixed with a pipette ten times. The solution was transferred to an RNeasy column, and centrifuged for 15 s at 15800 x g. The flow through was discarded and the column was washed using the manufacturers' buffers. The column was centrifuged for 2 minutes at 15800 x g to remove any excess ethanol before eluting the RNA with 40 μl of RNase free water and storing at -80 °C. RNA was quantified with UV spectrometry (see section 2.10).

2.10 Quantification of nucleic acids

The concentration of RNA and cDNA was calculated by measuring the optical density (O.D.) of the nucleic acid in question using UV spectroscopy. One O.D. unit is equivalent to 40 μg ml^{-1} single stranded RNA and 33 μg ml^{-1} single standed cDNA. Sample nucleic acid (1μl) was diluted into 100 μl ddH_2O and the absorbance read at 260 and 280 nm on a UV spectrophotometer. The

absorbance ratio of 260 nm / 280 nm was used as a measure of purity. A ratio of 1.8 – 2 was seen as an acceptable level of protein contamination.

2.11 Quantitative PCR

2.11.1 First strand cDNA synthesis

RNA samples (3 μg) were placed into 500 μl PCR tubes and incubated with 0.5 μl random primers (500 ng ml^{-1}) at 70 °C for 10 minutes then cooled to 25 °C for 2.5 minutes. The contents of table 2.3 were then added and the samples incubated at 25 °C for a further 2.5 minutes. The samples were then incubated at 50 °C for 90 minutes, 70 °C for 10 minutes and then cooled to 37 °C for 1 minute. 0.5 U RNase H (USB Europe GmBH, Staufen, Germany) was added and the samples incubated at 37 °C for a further 29 minutes. Samples were purified using the Qiagen PCR clean up Kit (see section 2.11.2).

Table 2.3 Preparation of first-strand reaction mixture

	vol / μl
5x reaction buffer	4
0.1 M DTT	2
10 mM (each) dNTPs	1
40 U/μl RNase out	0.6
200 U/μl Superscript III	1

2.11.2 Purification of cDNA

cDNA was purified using a PCR clean up kit (Qiagen, UK). The reaction mixture was mixed with 5 volumes of buffer PB and loaded onto the column. The column was centrifuged at 18000 x g to bind the cDNA. The column was washed with 0.75 ml buffer PE and centrifuged at 15800 x g for 1 minute. The

eluant was removed and the column centrifuged at 15800 x g for 2 minutes to dry the column. The cDNA was eluted in 30 µl of buffer EB (10 mM Tris/HCl pH 8.5) and stored at -20 °C. The cDNA was quantified using UV spectrometry (see section 2.10).

2.11.3 Real-Time PCR

Real-time PCR was used to measure the abundance of particular mRNAs from treated cell lines. The primer/probe sets used were either commercially bought (Assays on demand, Applied Biosystems, UK) or designed in-house using a primer design program (Primer Express, Applied Biosystems, UK) and manufactured by Eurogentec (UK). The assays for the genes of interest were matched with assays for the loading controls as follows: Bcl-x_L, GAPDH; IAP, β-actin; Bak, PRP2; MCT1, PRP2. The matched assays were then performed in a multiplex reaction with the abundance of mRNA for the gene of interest and the loading control being determined at the same time.

Each PCR reaction was prepared in a 20 µl volume and consisted of: 12.5 µl reaction buffer (Taq-Man Jump start ready mix, Sigma, UK), 1.25 µl gene of interest primers and probe assay mix (900 nM final concentration), 1.25 µl loading control primers and probe assay mix (300 nM final concentration) and 5 µl ddH_2O. 5 µl of sample cDNA (10 $ng\mu l^{-1}$) or ddH_2O (for no template controls) was then added. The cycling parameters for the reaction were: an initial 2 mins at 95 °C to activate the DNA polymerase followed by 95 °C, for 15 seconds and 60 °C for 1 minute repeated for approximately 60 cycles. The PCR was carried out on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) and analysis performed using the Rotor-Gene 3000 computer software (Corbett Research, Australia).

2.12 Northern Analysis

2.12.1 Agarose gel electrophoresis of RNA

RNA samples were resolved using 1 % (w/v) agarose denaturing gels. 0.4 g agarose was added to 33.8 ml ddH_2O and 4 ml 10x MOPS (0.2 M MOPS, 50 mM sodium acetate, 5 mM EDTA, pH 7), and heated in a microwave to dissolve the agarose. The solution was left to cool to 50 °C (hand hot) to which 2 ml formaldehyde was added. The gel was poured into a gel rig (Hoefer Inc, San Francisco, CA, USA) and left to set at room temperature for 30 minutes. RNA samples (10 µg) were diluted 1:1 with RNA denaturing sample buffer (50 % (v/v) formmide, 20 % (v/v) formaldehyde, 20 % (v/v) 10x MOPS) and heated to 65 °C for 5 minutes before cooling on ice for 2 minutes. 6x RNA loading dye (50 % (v/v) glycerol, 1 mM EDTA, 0.4 % (w/v) bromophenol-blue, 0.4 % (w/v) xylene cyanol) was added to each sample before loading on to the gel. High range RNA markers (Fermentas International Inc, Burlington, Ontario, Canada) were used and treated in the same regard as the RNA samples above. Electrophoresis was carried out at 80 volts until the bromophenol-blue dye reached the end of the gel.

2.12.2 Transfer of RNA to nitrocellulose membrane

After electrophoresis, the gel was rocked in ddH_2O for 15 minutes to reduce the amount of formaldehyde. The RNA was transferred to nitrocellulose membrane (Hybond N+, Amersham Pharmacia Biotech, UK) using capillary action. Into a plastic dish went the following: sponges, 3 pieces of 3 MM paper, the RNA gel upside down, the membrane, 2 pieces of 3 MM paper, paper towels, a glass plate and a 0.5 kg weight. The RNA was left to transfer over

night in 10x SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0). Following transfer the RNA was fixed to the membrane by UV cross-linking (UV Stratlinker-2400, Stratagene, UK). Transfer efficiency was assessed by staining the membrane with a methylene-blue reversible stain (0.02 % (w/v) methylene blue, 0.3 M sodium acetate, pH 5.5) and destained using a buffer consisting of 0.2x SSC and 1 % (w/v) SDS. The membranes were either used on the same day or stored at -20 °C until further use.

2.12.3 Hybridisation of radiolabelled probe to the northern membrane

The membranes were pre-wetted with 2x SSC before pre-hybridising at 42 °C for 3 hours with hybridisation solution (25 mM sodium MES, 2.5 mM sodium pyrophosphate, 50 % (v/v) deionised formamide, 10 % (w/v) dextran sulphate, 5x SSC, 3x Denhardt's (1 % (w/v) BSA, 1 % (w/v) PPP, 1 % (w/v) Ficoll® 400), 0.2 % (w/v) SDS, 0.1 % (v/v) Antifoam B (Sigma-Aldrich, UK)) in a rotating hybridisation oven. The membranes were incubated at 42 °C for 16 hours in the same buffer containing a radiolabelled cDNA probe. Labelling was carried out with [α-^{32}P]-dCTP using a HexaLabel Oligolabelling kit (Amersham Pharmacia Biotech, UK). Following incubation the membranes were washed with increasing stringency to remove unbound probe. Low stringency washes were carried out with 5x SSC, 1 % (w/v) SDS and 0.25 % (w/v) SARKOSYL at 42 °C; medium stringency with 1x SSC and 0.1 % (w/v) SDS at 42 °C; and higher stringency washes using 0.1x SSC and 0.1 % (w/v) SDS at 55 °C. Excess buffer was removed by dabbing the corner of the membrane on tissue paper. The membranes were wrapped in saran wrap and Tracker Tape attached (Amersham Ltd., UK) before subjecting to autoradiography.

2.12.4 Autoradiography

The wrapped membranes were placed into an X-ray cassette (Sigma, UK) fitted with a Trans-Screen (Anachem Ltd., UK) intensifying screen. The membranes were exposed to BioMax-MS film (Kodak, UK) at -80 °C from 12 to 48 hours before developing the film. The intensity of the bands was determined by scanning densitometry (Phoretix ID quantifier, Non-linear Dynamics).

2.12.4 Stripping and re-probing of the nitrocellulose membrane

In order to re-probe the membrane for another transcript of interest, the membranes were stripped by washing 3 times in a boiling solution of 0.1 x SSC and 0.1 % (w/v) SDS. The membranes were rinsed in 2 x SSC before carrying out hybridisation with another radiolabelled probe to the transcript of interest as described previously.

2.13 Statistical analysis

Statistical analyses were performed using a paired student t test. Results were considered to be significant (*) if a p-value ≤0.05 were obtained; very significant (**) if a p-value ≤0.01 were obtained and extremely significant (***) if a p-value of ≤0.001 were obtained.

CHAPTER 3

Synthesis of disodium poly(ethylene glycol)$_{600}$α,ω-di(4-butyrate)

3.1 Introduction

Coupling of biologically active compounds to membrane-impermeable molecules allows researchers to discriminate between the effects induced by biological active agents on the surface of plasma membranes, and intra-cellular effects resulting from entry into the cell. Chemical modification of biologically active compounds to produce membrane-impermeable analogues can be achieved through a variety of means. These include: production of charged derivatives (Marrero-Alonso *et al.*, 2006; Zhang *et al.*, 1999), increasing the hydrophobicity of the compound through methylation (Wittekindt *et al.*, 2006) and coupling the molecule of interest to a larger compound that is already membrane-mpermeable. In the latter case, the choice of membrane impermeable compound can also be varied and specific examples include: acetyl co-enzyme A (Dutta *et al.*, 2002); biotin, either alone (Mahfoud *et al.*, 2006), or as biotin derivatives (Pardridge, 1999; Pavlov & Glaser, 2002), the protein bovine serum albumin (Arima, 2006; Gatson *et al.*, 2006) and polyethylene glycol (Lee *et al.*, 2003) .

Polyethylene glycol (PEG) is a long chain polymer of repeating ethylene oxide groups that have the general formula of $HO\text{-}(CH_2CH_2O)_n\text{-}H$ with n ranging from 6 to 1000. PEGs are molecules which appear as transparent oils in the low molecular weight range to waxy solids in the high molecular weight range and can withstand high temperatures and pressures. The ethylene oxide back-bone of PEG allows the molecule to be soluble in aqueous as well as protic and aprotic organic solvents such as acetone, benzene, chloroform, dichloromethane, dimethylsulphoxide, ethanol and toluene (Hinds & Kim, 2002; Kodera *et al.*, 1998; Zalipsky, 1995).

PEG is relatively inert biologically and its low immunogenicity and non-antigenic properties have attracted the interest of many researchers. The use

of PEG in PEG-compound conjugates, refered to as PEGylation, is a vast and growing field with the largest focus being on protein PEGylation (Fee & Van Alstine, 2006; Kodera et al., 1998; Roberts et al., 2002; Veronese & Pasut, 2005; Zalipsky, 1995). PEGylation involves the coupling of single or multiple PEGs to biologically active compounds which then take on the properties of the PEG. The resulting compounds have increased molecular weights reducing the amount of the compound subjected to the reticuloendothelial system of the spleen, liver and kidneys; thereby increasing the circulation in the blood and so too the drugs half-life (Hinds & Kim, 2002; Xu *et al.*, 2005; Yu *et al.*, 2005). The low immunogenicity and non-antigenic properties of PEG are transferred to the compound of interest during PEGylation, lowering the host animal's immune response towards the compound of interest (Roberts *et al.*, 2002). Indeed, The Food and Drug Administration (FDA) in The United States of America have approved PEG for human internal consumption and there are several drug-PEG conjugates on the market. These include: PEG-asparaginase (Oncaspar®), PEG adenosine deaminase (Adagen®) and PEG α-interferon (PEG-intron®) (Veronese & Pasut, 2005; Fee & Van Alstine, 2006). PEG has been used in biochemical research as a membrane impermeable anchor in many PEG-compound conjugates including: radioimmunoimaging of liver metastases (Li *et al.*, 2006); proteins, camptothecin (Yu *et al.*, 2005) and superoxide dismutase (Wang *et al.*, 2006); and the drug ibuprofen (Davaran *et al.*, 2006). Previous work carried out in this laboratory involved the synthesis of a PEG-glucose conjugate, α,ω-di(glucos-6-yl)PEG_{600}. α,ω-di(glucos-6-yl)PEG_{600} was subsequently used in *in vivo* and *in vitro* studies to help identify and characterise a glucose sensor in the small intestine (Dyer *et al.*, 2003). α,ω-di(glucose-6-yl)PEG_{600} was synthesised according to the scheme in Figure 3.1 and involved a three step synthesis. The hydroxyl groups on the end of

PEG were first halogenated using CBr_4 in CH_2Cl_2 before coupling the halogenated PEG with methylated D-glucose in DMSO. The resulting product, α,ω-di(methylglucos-6-yl)PEG_{600} was hydrolysed under acidic condition in 4 M H_2SO_4 to produce α,ω-di(glucos-6-yl)PEG_{600}.

Under the conditions outlined by Dyer *et al.*, (2003) the methyl D-glucose was used as the nucleophile to replace bromine/chlorine from the halogenated PEG. Based on the work of Dyer *et al.*, (2003), a two step procedure was developed as outlined in the scheme in Figure 3.2, for the synthesis of a PEG butyrate conjugate, disodium $PEG_{600}\alpha,\omega$-di(butyrate). PEG was used in its

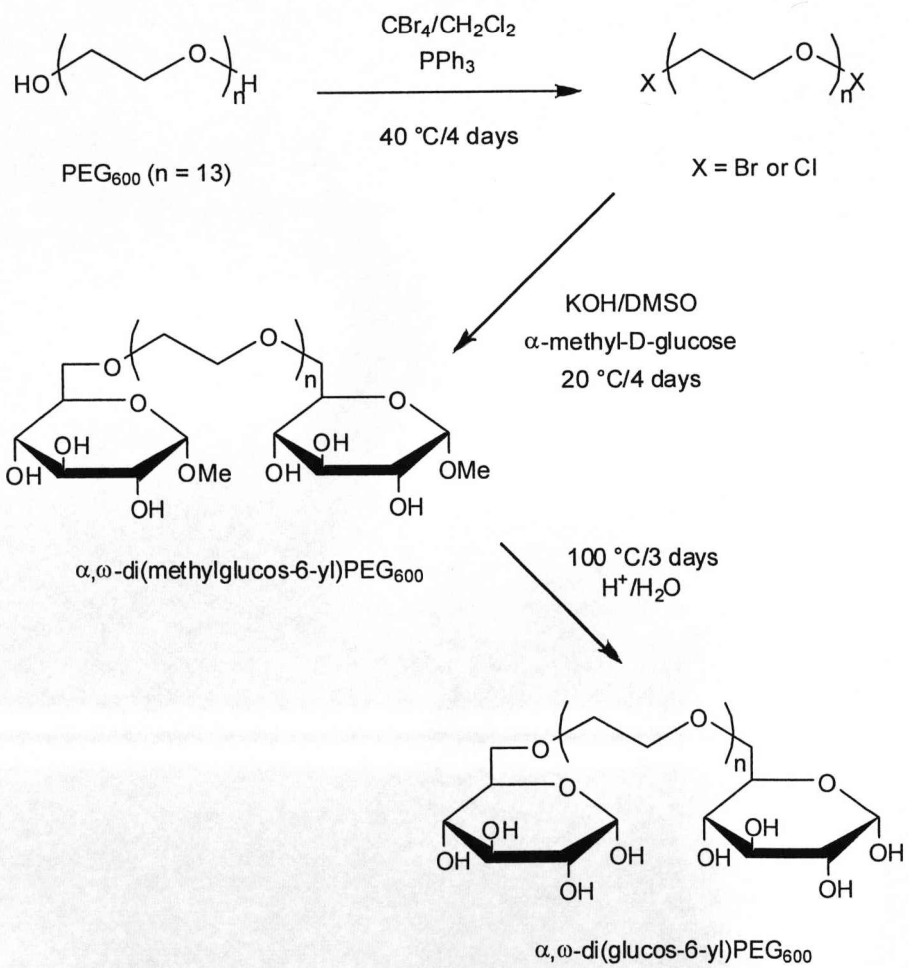


Figure 3.1 Schematic illustrating the synthesis of di(glucose-6-yl)PEG_{600}

standard form, with the hydroxyl groups acting as nucleophiles. Bromobutyronitrile was used as the substrate, eliminating the need to brominate the PEG and reducing the synthesis to a two step process. Bromobutyronitrile was used instead of bromobutyric, acid to prevent any by-product from a reaction with the carboxylic acid moiety (lactonisation). Once coupled to the PEG, the butyronitrile can be hydrolysed to butyric acid under either acidic or basic conditions.

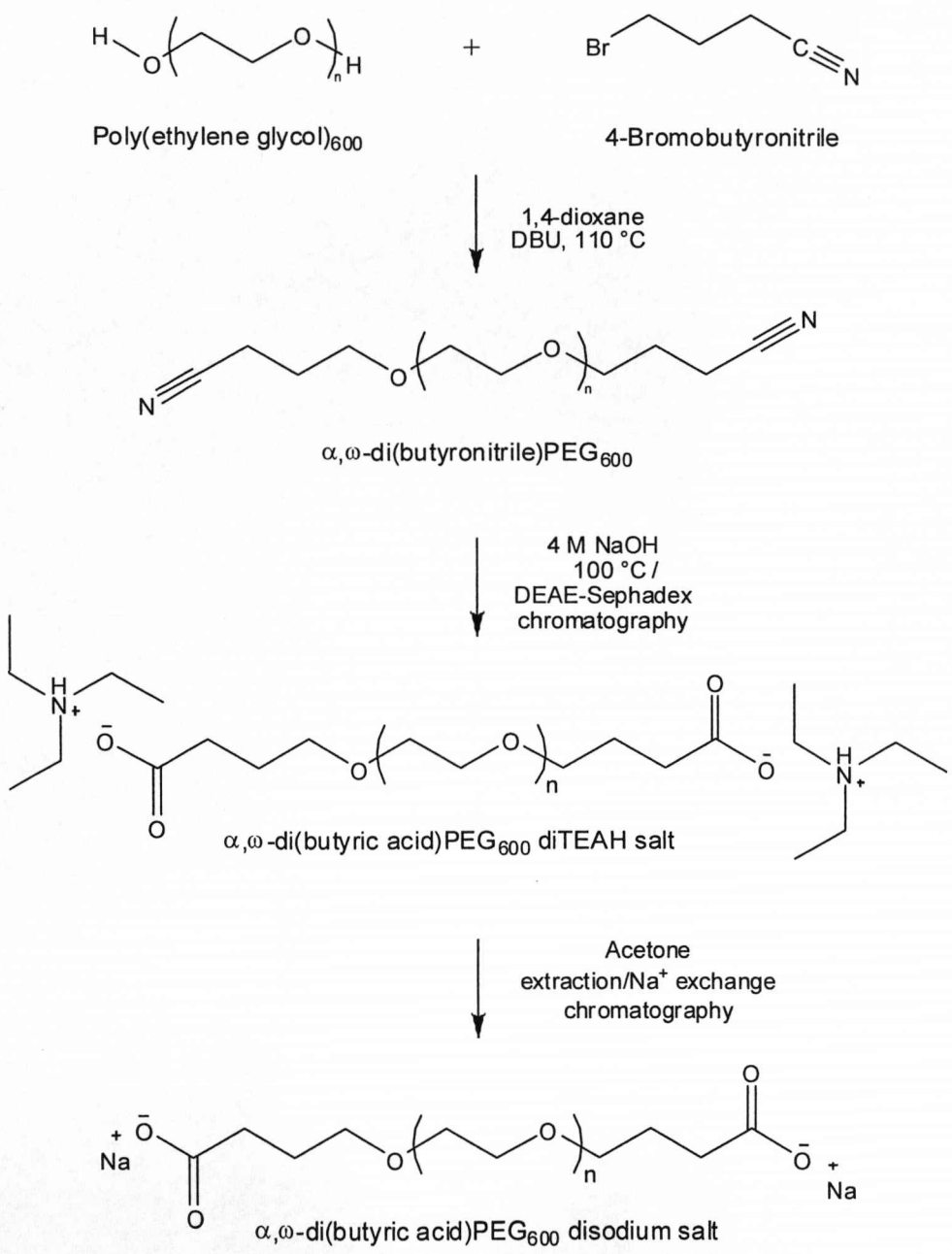


Figure 3.2 Schematic illustrating the synthesis of disodium PEG_{600} α,ω-di(4-butyrate)

3.2 Results

3.2.1 The synthesis of di(4-butronitrile)PEG_{600}

4-Bromobutyronitrile was added to a stirred solution of PEG_{600} and DBU in 1,4-dioxane and left to stir at 110 °C for a total of 4 weeks. The reaction was initially followed for 2 months by TLC. Staining of the TLC plates was carried out with iodine vapour and K_2MnO_4, using separate plates for each stain. The loss of PEG_{600}, which produced a large elongated spot (average R_f = 0.26), and the formation of α,ω-di(4-butyronitrile)PEG_{600}, which produced a small discrete spot (average R_f = 0.72) were followed by TLC. After the approximately 3 months, TLC showed complete loss of PEG_{600}. The reaction was left to stir for a further 4 weeks while the formation of di(4-butyronitrile)PEG_{600} was confirmed by mass spectrometry. However PEG_{600} was also present on the mass spectra with (4-butyronitrile)PEG_{600}. The reaction time, albeit very long, was in part due to the scheduling of the chemical synthesis around biochemical work being carried out in parallel (see chapter 5) that was favourable at the time.

3.2.2 Hydrolysis of di(4-butronitrile)PEG_{600}

In order to generate the carboxylic acid, the nitrile group was hydrolysed under basic conditions. The crude $PEG_{600}\alpha,\omega$-di(4-butyronitrile) was added to 20 ml 4 M NaOH and left to stir at 100 °C for 4 weeks. The formation of α,ω-di(4-butyric acid)PEG_{600} was followed using TLC, that gave a small discrete spot just above the base line of the TLC plate with an average Rf = 0.02. Once hydrolysis of α,ω-di(4-butyronitrile)PEG_{600} was complete, as determined by mass spectrometry of the crude reaction mixture, the solution was neutralised

with 1 M HCl and concentrated before purifying with diethylaminoethyl (DEAE)-Sephadex.

3.2.3 DEAE-Sephadex chromatography

The hydrolysis of α,ω-di(4-butyronitrile)PEG_{600} had taken place without any prior purification. Anion exchange chromatography was selected for the purification media as this would give specific purification of products containing a carboxylic acid function.

The crude product was concentrated to approximately 5 ml and added onto a DEAE-sephadex (bicarbonate form) column equilibrated with distilled water. The column was washed with distilled water until the filtrate ran clear. TLC confirmed the present of DBU and PEG_{600} in the distilled H_2O fractions. The α,ω-di(4-butyric acid)PEG_{600} was then eluted with 1 M triethylammonium bicarbonate (TEAB) to give the product as the triethylammonium salt. The fractions containing α,ω-di(4-butyric acid)PEG_{600}, as determined by TLC, were pooled and dried by rotary evaporation to yield a pale yellow solid. ^{1}H NMR determined this to be a mixture of triethylammonium (TEAH) salts and α,ω-di(butyric acid)PEG_{600}.

The TEAB contaminant was removed using a series of acetone extractions. Thus solid TEAH α,ω-di(4-butyric acid)PEG_{600} mixture was stirred in acetone to dissolve the α,ω-di(4-butyric acid)PEG_{600} and filtered to remove the undissolved TEAB salts. The TEAB α,ω-di(4-butyric acid)PEG_{600} was then washed several times, each wash reducing the amount of TEAB present. The level of TEAH present was continually followed by ^{1}H NMR until a required stoichiometric ratio of TEAH and α,ω-di(4-butyric acid)PEG_{600} was observed.

3.2.4 Na^+ exchange chromatography

Final purification of α,ω-di(4-butyric acid)PEG_{600} was carried out with sodium exchange chromatography resulting in the isolation of the sodium salt of the butyric acid. Cellex-P exchange resin, hydrogen ion form, was stirred in 0.5 M NaOH for 15 minutes to produce the Na^+ ion form. The resin was poured into a column and washed 3 times with dH_2O before loading the concentrated α,ω-di(4-butyrate)PEG_{600} triethylammonium salt onto the column. The α,ω-di(4-butyric acid)PEG_{600} was eluted from the column using distilled water and dried using a rotary evaporator with a "cold finger", to give the disodium PEG_{600}α,ω-di(4-butyrate) as a brown oil.

3.3 Discussion

3.3.1 The synthesis of di(4-butronitrile)PEG_{600}

The reactions conditions first employed in the synthesis of α,ω-di(4-butronitrile)PEG_{600} (PEG-BN) were based on the approach outlined by Dyer *et al.*, (2003) in the synthesis of α,ω-di(glucose-6-yl)PEG_{600}, a membrane impermeable glucose analogue. In the Dyer approach, polyethylene glycol 600 was first brominated then reacted with methylated D-glucose in an S_N2 reaction. Under the reaction conditions, the methylated D-glucose reacted by a nucleophilic attack on the brominated PEG, displacing bromine for methylated D-glucose.

For the synthesis of α,ω-di(4-butyronitrile)PEG_{600} (PEG-BN), PEG was used in its unmodified form with hydroxyl groups intact, reversing the role of PEG from the electrophile to the nucleophile. In this case, PEG acting as the nucleophile, makes a nucleophilic attack on the fourth carbon of bromobutyronitrile, displacing bromine, to form an ether linkage. By using PEG as the nucleophile and a commercially available brominated substrate, the synthesis was reduced to a two stage process. The remaining reaction conditions outlined by Dyer et al., (2003) were followed; these included dimethyl sulphoxide (DMSO) as solvent, potassium hydroxide as base and stirring the reaction mixture at room temperature in the dark for approximately four days for each step. The starting and revised reaction conditions for the synthesis of α,ω-di(4-butyric acid)PEG_{600} can be seen in figure 3.3; figures 3.3A1 and 3.3B1 show the changes made in the synthesis of α,ω-di(4-butyronitrile)PEG_{600}.

Following the KOH/DMSO conditions employed by Dyer et al., (2003), resulted in a prolonged reaction with no product forming during several days after

starting the reaction. After 4 weeks had elapsed, some reaction between the bromobutyronitrile and polyethylene glycol had taken place, however a large amount of PEG_{600} was still present, as seen by TLC and mass spectrometry. The stoichiometry of the reaction was increased from a ratio of 1:3 to a ratio of 1:6 (PEG:bromobutyronitrile) in an attempt to drive the reaction to completion. The increased amount of bromobutyronitrile in the reaction brought about a complication. Bromobutyronitrile is a liquid at room temperature and soluble in a water:DMSO (1:1, v/v) mixture. When the ratio of PEG to bromobutyronitrile was increased to 1:6 the volume of bromobutyronitrile present was too great to dissolve in the water:DMSO mixture and produced an organic layer thereby creating a two phase system. Stirring the reaction had no effect on mixing of the two layers, which separated immediately when stirring ceased. The water:DMSO mixture was used as a convenient method for the addition of KOH into the reaction. 2 M Potassium hydroxide was diluted 1:1 with DMSO, resulting in a 50 % aqueous solution. The hydroxyl ions introduced by water into the reaction mixture have a greater reactivity than the hydroxyl groups on alcohols such as PEG and can attack the bromobutyronitrile resulting in hydroxybutyronitrile (Clayden *et al.*, 2001). This added to the problems caused with the BBN failing to dissolve in the water/DMSO solution. In an attempt to resolve this, potassium hydroxide was crushed under nitrogen

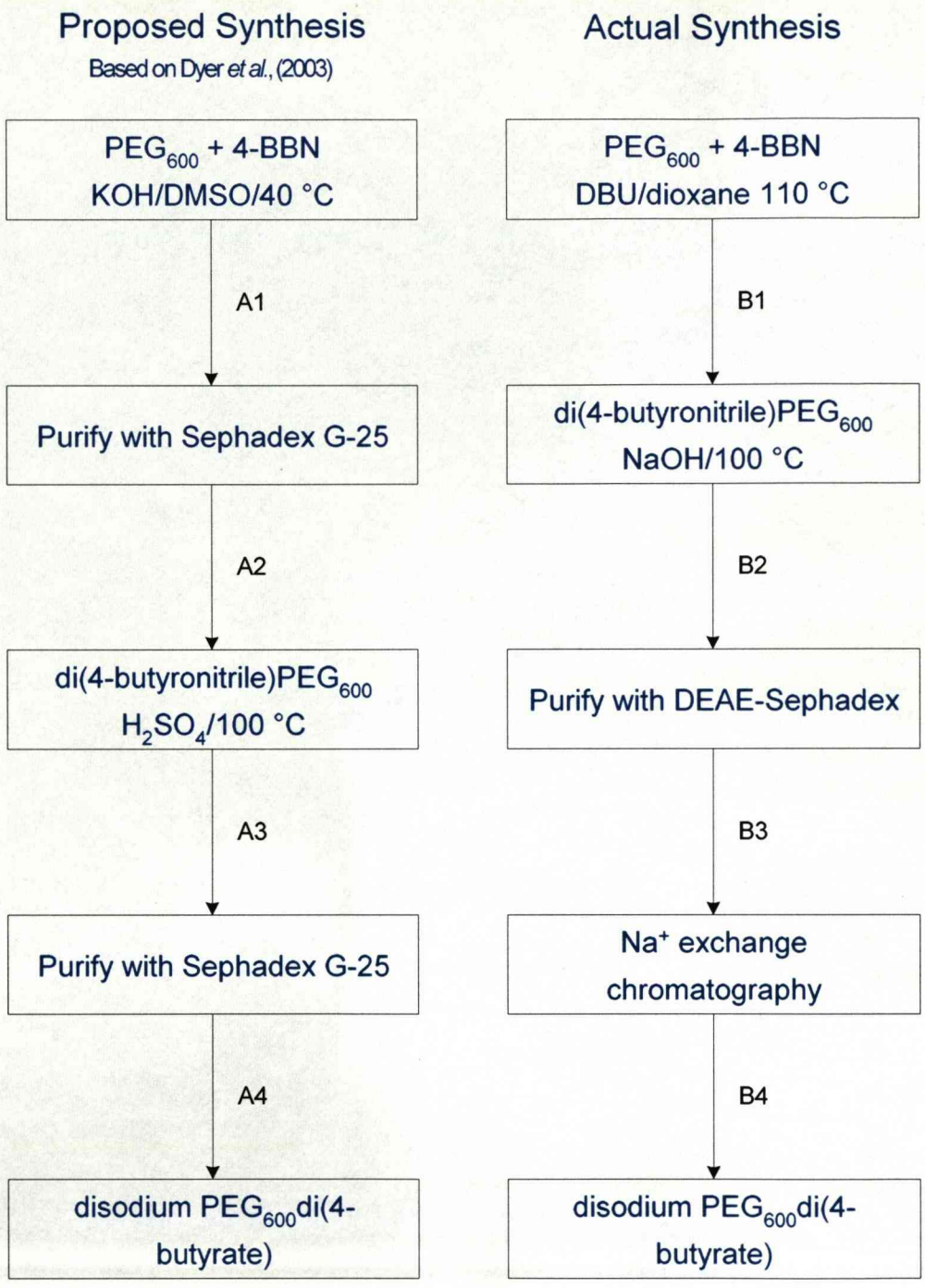


Figure 3.3 Schematic outlining the synthesis of disodium $PEG_{600}\alpha,\omega$-di(4-butyrate)

and added directly to dry DMSO, thus limiting the availability of water in the reaction and allowed the BBN to fully dissolve.

DMSO has a boiling point of 189 °C and has low volatility. As a result, DMSO was retained on the TLC plates and chromatographs to give anomalous results. Accordingly the solvent was changed from DMSO to 1,4-dioxane (dioxane). Dioxane was chosen as it is a good aprotic organic solvent with a lower boiling point of 101.5 °C. Dioxane also has a low volatility but did not produce any discrete isolated spots on the TLC plates; however, it did elongate the spots produced from other compounds. Crucially no anomalous results were seen on the TLC plates when dioxane was used as the solvent. However, the solubility of potassium hydroxide in dioxane is considerable less than in DMSO and problems with dissolving the potassium hydroxide arose. Indeed, after 24 hours of stirring approximately half the potassium hydroxide had failed to dissolve in the dioxane and formed a solid layer at the bottom of the flask.

Added to the problems with dissolving the potassium hydroxide in dioxane, the potassium ions were carried over in a work-up and purification of the di(4-butyronitrile)PEG_{600}. A strong base is required in the reaction in order to deprotonate the hydroxyl groups on the PEG, thus preparing the PEG alkoxide ion that will subsequently react with 4-BBN (Clayden *et al.*, 2001). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) is an organic compound which is a strong base, has low nucleophile properties and fully dissolves in dioxane. (see figure 3.2 A1 and 3.2B1)

Overall the reaction between PEG and 4-BBN was very slow. The rates of S_N2 reactions between primary alcohols and primary alkyl halides can be fast. However, the size of the nucleophile and substrate, the solvent used and temperature of the reaction all have an effect on the rate and yield of the reaction. Hydroxyl groups do not make good leaving groups in an S_N2 reaction and are more often converted to a better leaving group; for the synthesis of

di(methylglucos-6-yl)PEG_{600} the hydroxyl group was converted to a halogen. Halogens make good leaving groups, with an iodide ion being the best leaving group and a fluoride ion the poorest. The general order in ease of leaving is as follows: $I^- > Br^- > Cl^- >> F^-$ (March, 1985). Small, charged nucleophiles are solvated less in aprotic solvents compared to protic solvents, allowing more contact between the nucleophile and the solvent. However this solvent effect becomes less important as the size of the substrate and nucleophile increase (March, 1985). Here steric effects become more important in affecting rate and yield of a reaction.

The choice of nucleophile in this synthesis might have an important effect on the reaction. Bulky nucleophiles are less reactive than small nucleophiles, due to the nonbonded repulsions that develop during the S_N2 transition state (Carey & Sundberg, 1990). While PEG is a primary alcohol, the chain length of PEG is considerably long which could have had a significant effect on the rate of a reaction. The rate of an S_N2 reaction generally decreases as the carbon chain length of the nucleophile increases. PEG has a chain length of between 18 and 45 (counting the CH_2-CH_2-O repeating monomer of the PEG backbone) making it considerable larger than the substrate it is attacking.

3.3.2 Workup and purification of PEG-BN

In order to simplify the purification of the end product, removal of any unreacted PEG, bromobutyronitrile and DBU is desirable before continuing on to hydrolyse the α,ω-di(4-butyronitrile)PEG_{600}. Column chromatography using Sephadex G-15 media was first used, as this was the method of choice by Dyer *et al.*, (2003) Sephadex G-15 is a size-exclusion solid media whose matrix excludes molecules above 1500 mw. When synthesising α,ω-di(glucose-6-yl)PEG_{600}, the reactions conditions were set up to ensure that the

reaction went to completion and resulted in a very small amount of starting material present, with a yield of 98 %. The purpose of purification using Sephadex G-15 was therefore to remove excess K^+ salts. Here however, starting material was still present. When purifying using Sephadex G-15, separation of the K^+ salts from the PEG-BN was expected due to the size difference between the two species. However, some K^+ salts were eluted in the fractions containing the PEG-BN together with some unreacted PEG thereby making purification of the PEG-BN extremely difficult.

During the initial reactions when DMSO and potassium hydroxide were used as the solvent and base respectively, purification with Sephadex G-15 was used. As the reaction conditions were changed, purification with Sephadex proved even more difficult. Changing the base from potassium hydroxide to DBU also brought more complications as the DBU eluted with the PEG-BN, resulting in a highly contaminated product. Increasing the amount of BBN to excess also meant that there was unreacted BBN in the reaction mixture that failed to separate from the PEG-BN when using Sephadex G-15. Using column chromatography to purify PEGs and PEG conjugates is a notoriously difficult procedure to master due to the heterogeneous mixture of polymers that make up PEG, (Fee & Van Alstine, 2006). Mass spectrometry showed a range of polymer chains from 400 to 1000 $gmol^{-1}$. It was therefore decided to take the reaction mixture containing PEG-BN and use it directly in the hydrolysis reaction.

3.3.3 Hydrolysis of PEG-BN under acid and basic conditions

Hydrolysis of PEG-BN involves the hydrolysis of the nitrile initially to the amide and then to the carboxylic acid. Hydrolysis can occur under either acid or base catalysis. In order to establish which method to use, both acid- and base-

catalysed reactions were examined using a small amount of the PEG-BN. In the trial reactions 0.5 g PEG-BN was placed into a 10 ml round bottom flask containing either 4 M HCl or 4 M NaOH and stirred at 50 °C (see Figure 3.3A3 and 3.3B2). After approx. 2 weeks the acid-catalysed reaction failed to form appreciable quantities of the PEG-BN as seen by TLC. During the same period TLC of the base-catalysed reaction indicated that it had gone to completion. However, there was some of the intermediate amide present as seen by mass spectrometry. After a further 2 weeks the intermediate butyramide was still present. Increasing the temperature of the reaction to reflux produced a mixture of the mono-acid, mono-acid and amide or di-acid PEG-conjugates, as assessed by mass spectrometry. No peaks corresponding to the mono or di-substituted α,ω-(4-butyronitrile)PEG_{600} were seen on the mass spectra confirming that hydrolysis of the nitrile group had taken place.

The reaction times reported for base-catalysed reaction include the time taken to assess progression of the reaction via mass spectrometry. All mass spectrometry was carried out in house by the mass spectrometry service where there were delays between submitting and receiving mass spectra. As the PEG-BN was not purified after synthesis, the exact amount present in the hydrolysis reaction was not known and consequently, the yield for the hydrolysis reaction can only be estimated. As no mono- or di-butronitrilePEG_{600} products were seen by mass spectrometry, it is estimated that the hydrolysis of α,ω-di(butyric acid)PEG_{600} is $\geq$ 95 %.

3.3.4 Anion Exchange Chromatography

Purification of α,ω-di(4-butyric acid)PEG_{600} was carried out using DEAE-sephadex. The DEAE residues bind negatively charged species which makes this an ideal method to purify α,ω-di(4-butyric acid)PEG_{600} whose carboxylic

residues will bind to the DEAE groups. Any unreacted starting material, DBU and dioxane will be washed through the column. Elution of the product from the column was carried out using TEAB with an initial gradient of 0 – 0.3 M TEAB, The elution of α,ω-di(4-butyric acid)PEG_{600} was followed using TLC, using a bromocresol green stain with α,ω-di(4-butyric acid)PEG_{600} being eluted in the fractions that proceeded the addition of TEAB onto the column.

α,ω-Di(4-butyric acid)PEG_{600} was not completely eluted from the column using 0.3 M TEAB; increasing the gradient to 0.5 M TEAB also proved unsuccessful in eluting all the α,ω-di(4-butyric acid)PEG_{600} as seen by the present of α,ω-di(4-butyric acid)PEG_{600} in the last fraction eluted from the column. The gradient was thus increased to 1 M TEAB whereby all α,ω-di(4-butyric acid)PEG_{600} was eluted from the column. Subsequent batch purifications did not use a TEAB gradient; after washing the DEAE-sephadex column with distilled water, α,ω-di(4-butyric acid)PEG_{600} was eluted using 1 M TEAB reducing the total volume of TEAB required.

Purification of the α,ω-di(4-butyric acid)PEG_{600} with DEAE-sephadex resulted in a yellow solid that was, according to theoretical yield calculations, 4 times greater than expected. ^1H NMR analysis confirmed the presence of a large amount of TEAH salt present in the yellow solid. Elution of acid products from DEAE-sephadex columns necessarily results in the TEAH salt of the acid. Any excess TEAH can be removed by repeated evaporation from water. In this case though, the amount of TEAH salt present was so great, that the volume of distilled water required made removal of the TEAH salt a slow and tedious process. Several co-evaporations of the α,ω-di(4-butyric acid)PEG_{600} salted product with dH_2O did not make a significant impact on the amount of TEAH present. Removal of TEAH ions using column chromatography on silica was examined, but rejected due to the polar nature of the carboxylic acid groups in

the product. A search for alternative methods for the removal of TEAH was subsequently performed.

Oishi *et al.* prepared PEGylated antisense oligonucleotides and used isopropanol precipitation as the purification method. The PEGylated antisense olidonucleotides were prepared using 4 kDa PEGs in THF and purified by pipetting the reaction mixture into cold (-15 °C) propan-2-ol (Oishi *et al.*, 2005). The PEGylated antisense oligonucleotides precipitated out in propan-2-ol and were collected via centrifugation. The isolation of α,ω-di(4-butyric acid)PEG_{600} using isopropanol precipitation was investigated. The solid α,ω-di(4-butyric acid)PEG_{600} TEAH salt mixture was dissolved into THF and added to isopropanol (cooled to approximately -15 °C in a NaCl-acetone-ice bath) whereby precipitation occurred. The α,ω-di(4-butyric acid)PEG_{600} did not precipitate as a solid wax but rather as a semi-solid liquid that would mix with the isopropanol upon attempted removal of the isopropanol. The semi-solid nature of the α,ω-di(4-butyric acid)PEG_{600} is perhaps, not unexpected, due to its substantially lower molecular weight (~ 600 Da) as compared to the 4 kDa PEG used by Oishi *et al.* Although the isopropanol precipitation failed to adequately purify the α,ω-di(4-butyric acid)PEG_{600}, the TEAH ions did however dissolve in the isopropanol, and this presented the idea of using a solvent that would selectively dissolve the α,ω-di(4-butyric acid)PEG_{600} in the presents of the TEAH salts.

Thus crude TEAH α,ω-di(4-butyric acid)PEG_{600} product was stirred in a large excess of acetone (approximately 400 ml per 4 g) for 1 hour. The acetone, now yellow in colour, was filtered through Whatman paper and evaporated to yield a brown oil. The brown oil was further stirred in acetone to remove more TEAH salt, which was collected and subjected to further acetone washes in an attempt to extract as much α,ωdi(4-butyric acid)PEG_{600} as possible. Figure. 3.4

illustrates the stages involved in extracting the α,ω-di(4-butyric acid)PEG_{600} from the TEAH salt. The removal of TEAH was continually followed with ^1H NMR. The TEAH ions showed two distinct peaks; a triplet at 1.28 ppm, representing the terminal methyl group and a quartet at 3.25 ppm, representing the methylene group adjacent to nitrogen. The removal of TEAH continued until the amount of TEAH ions present was reduced to a minimum as determined by comparing the corresponding peaks present of TEAH and butyric acid on the ^1H NMR spectra; this being approximately 10 washes. At this time the α,ω-di(4-butyric acid)PEG_{600} TEAH salt was converted to the sodium salt using sodium-exchange chromatography.

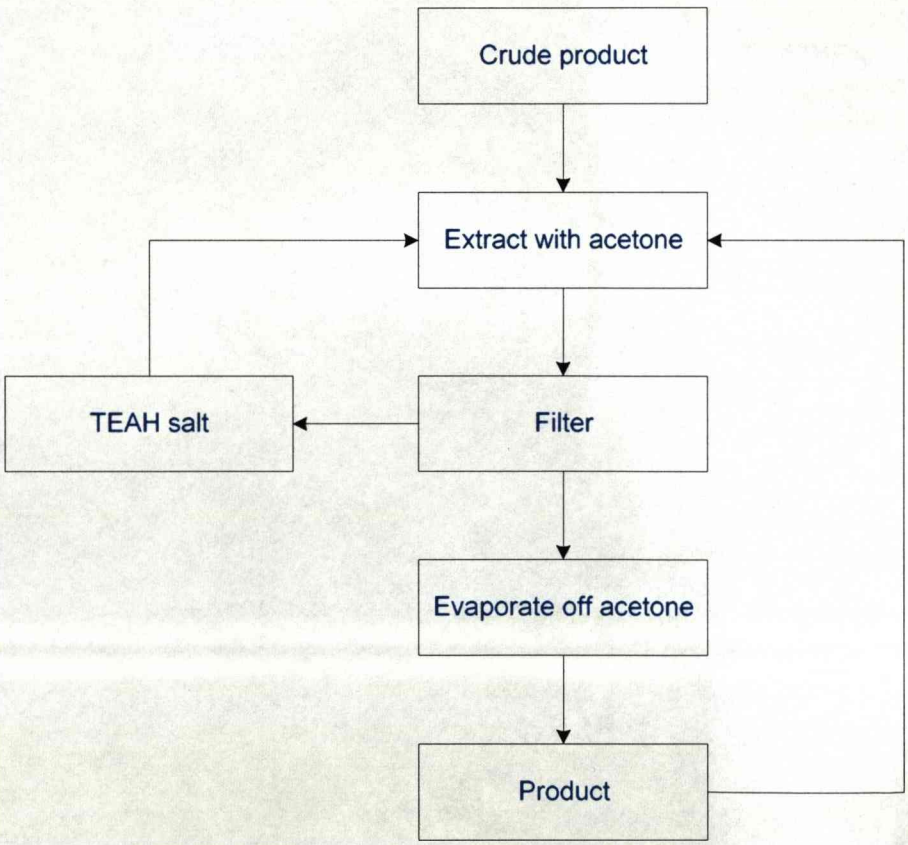


Figure 3.4 Schematic illustrating the removal of excess TEAH salt

4.3.5 Sodium-exchange chromatography

The exchange of TEAH for sodium ions was conducted in order to standardise the biological testing that was to be carried out using α,ω-di(4-butyric acid)PEG_{600}. The other substrates being tested were bought in in the form of sodium salts. TEAH α,ω- di(4-butyric acid)PEG_{600} was dissolved into approximately 4 ml distilled water and added onto the sodium-exchange resin. The α,ω-di(4-butyric acid)PEG_{600} was drawn through the column and eluted with distilled water. The water was evaporated off in vacuo to give a brown oil with a yield for disodium PEG_{600}α,ω-di(4-butyrate) of 13.2 %. ^{1}H NMR confirmed the absence of TEAH ions and butyronitrile. The peaks present on the ^{1}H NMR speactra that represented the butyric acid showed the correct $^3J_{HH}$ coupling; which was confirmed using 2D ^{1}H NMR correlated spectroscopy (COSY). As expected, no signals were seen on the ^{1}H NMR corresponding to the carboxylic acid group, which are expected to have a chemical shift around 11 ppm.

All expected signals for α,ω-di(4-butyric acid)PEG_{600} were seen on the ^{13}C NMR spectra, although the peak at 181 ppm representing the carboxylic acid carbon had a smaller peak than the corresponding peaks for C2-C4. Signals for C2-C4 of (4-butyronitrile)PEG_{600} were seen on the ^{13}C NMR but not the signal for the carbon of the nitrile group. Mass spectrometry confirmed the presence of the heterobifunctional PEG_{600} containing a butyric acid group and a butyronitrile group at either end of the PEG molecule. Signals were seen representing mono- and di-substituted (4-butyric acid)PEG_{600} on the mass spectra. No signals corresponding to PEG_{600} were seen by either ^{1}H NMR or mass spectrometry confirming that all the PEG_{600} had been removed by the anion exchange chromatography. No peaks relating to (4-butyronitrile)PEG_{600} or α,ω-di(4-butyronitrile)PEG_{600} were present on the ^{1}H NMR or mass spectra.

Furthermore, no peaks relating to α,ω-(4-butyronitrile,4-butyrate)PEG_{600} were seen on the ^{1}H NMR; therefore, the amount of the butyronitrile group present is likely to be less than 5 %.

The idea of coupling butyric acid to PEG is not a new suggestion. Wakselman *et al.*, (1990) synthesised PEG-dibutyrate analogues that were coupled to PEG via an ester bond. This strategy was based on the principle that the butyric acid would be released at the site of action through cleavage of the ester bond. Wakselman *et al.*, (1990) found that PEG_{1000} dibutyrate had very good anti-tumour activity; determined by inoculating mice with 180 TG Crocker sarcoma cells followed by treatment of the α,ω-di(butyrate)PEG_{1000}. The α,ω-di(butyrate)PEG_{1000} was injected 3 days preceding inoculation and had a protective effect on the mice compared to the control mice (Wakselman *et al.*, 1990).

Overall coupling of butyric acid to PEG_{600} through a stable ether linkage has proved successful. Extensive characterisation including ^{1}H and ^{13}C NMR, IR and mass spectrometry showed the formations of a mono- and di-substituted butyric acid conjugated PEG_{600}. The membrane-impermeable butyrate analogue can now be used as a tool in determining the mechanism for butyrate regulation of gene expression in the large intestine. Here *in vitro* testing of disodium PEG_{600}α,ω-di(4-butyrate) will be carried out on the colonic epithelial cell lines, AA/C1 and HT-29. Known butyrate responsive genes will be assayed to investigate the need for butyrate entry into the cell, in order for butyrate to modulate the expression of the genes assayed (see chapter 4).

CHAPTER 4

Treatment of human colonic epithelial cell lines with disodium $PEG_{600}\alpha,\omega$-di(4-butyrate)

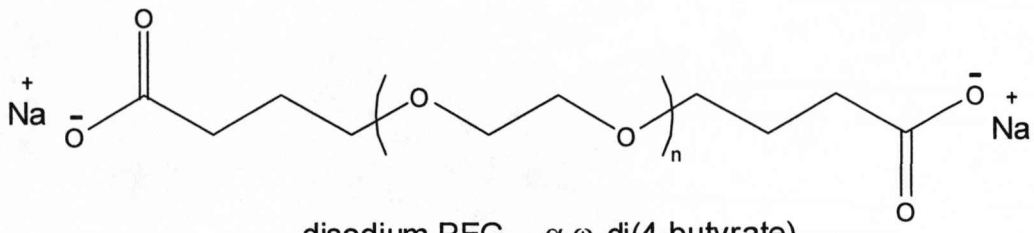
4.1 Introduction

Butyrate regulates a number of genes associated with differentiation, proliferation and apoptosis of colonic epithelial cells. Transport of butyrate across the colonocyte luminal membrane is mediated by MCT1 (Cuff *et al.*, 2002), the expression of which is dramatically down regulated during colon carcinogenesis (Lambert *et al.*, 2002). It has been proposed that the decline in MCT1 expression during colon carcinogenesis may reduce the intracellular availability of butyrate required to regulate expression of genes associated with the processes maintaining tissue homeostasis within the colonic mucosa. To test this hypothesis previous work in our laboratory employed the technique of RNA interference to specifically inhibit MCT1 expression, and determined the consequences of this inhibition for the ability of butyrate to exert its recognised effect *in vitro*. It was demonstrated, that inhibition of MCT1 expression, and hence butyrate uptake, has profound inhibitory effects on the ability of butyrate to regulate expression of key target genes; *CDKN1A* (p21), *ALP1* (IAP) and cyclin D1, and their associated processes of proliferation and differentiation. It was proposed that the ability of butyrate to induce cell cycle arrest and differentiation is dependent upon the transport of butyrate into the cell via MCT1 (Cuff *et al.*, 2005). In contrast, inhibition of MCT1 expression had no effect on the ability of butyrate to modulate the expression of either *bak1* or *bcl-x*$_L$, and this was reflected in a corresponding lack of effect on butyrate-induction of apoptosis. It was suggested that the potential mechanism by which butyrate regulates genes controlling apoptosis is independent of expression of MCT1. The proposed mechanism was that butyrate is sensed by a plasma membrane butyrate sensor expressed on the luminal membrane of colonocytes initiating a signalling pathway controlling the expression of genes such as *bak1* and *bcl-x*$_L$.

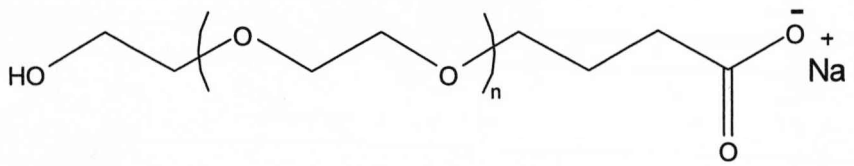
In the present study in order to assess the potential presence of the butyrate sensor and its effect on gene expression, a membrane impermeable butyrate analogue was synthesised. Butyrate was linked to poly(ethylene glycol)$_{600}$, the latter has been shown to be membrane impermeable (Lloyd, 1998) (See chapter 3 for the detailed synthesis). The aim was to treat colonic epithelial cell lines with butyrate and the membrane impermeable butyrate analogues, and determine their effects on the expression of candidate genes, *ALP1*, *CDKN1A*, *bak1* and *bcl-x$_L$*. To this end, the butyrate analogue disodium PEG$_{600}\alpha,\omega$-di(4-butyrate) was used in *in vitro* studies using two well-characterised human colonic epithelial cell lines, AA/C1 and HT-29 cells. Two cell lines were used in order to eliminate a single cell type specific effect. HT-29 are fast growing, poorly differentiated human colon adenocarcinoma cells which have been used as models of colonic epithelial cells. When exposed to butyrate these cells differentiate, and revert back to normality. In contrast AA/C1 cells are derived from a non-tumourigenic human colonic adenoma, are slow growing, and possess structural and functional properties modelling the differentiated absorptive cells of the normal colonic epithelium (Cuff *et al.*, 2002; Willams *et a.*, 1990).

During the synthesis of the butyrate analogue characterisation of the final product revealed that some butyronitrile was still present that had failed to be hydrolysed during synthesis (See chapter 3). The butyronitrile was determined to be part of a biheterofunctional PEG$_{600}$ with butyrate and butyronitrile coupled to either end of PEG molecule (sodium PEG$_{600}\alpha,\omega$-(4-butyrate,4-butyronitrile). Coupling of butyrate to PEG$_{600}$ did not result in the exclusive formation of di-substituted PEG, but a mixture of of mono- and di- substituted PEG$_{600}$(4-butyrate) as well as sodium PEG$_{600}\alpha,\omega$-(4-butyrate,4-butyronitrile), see figure 4.1 below. For simplicity the mixture of mono- and di- sodium PEG$_{600}$(4-

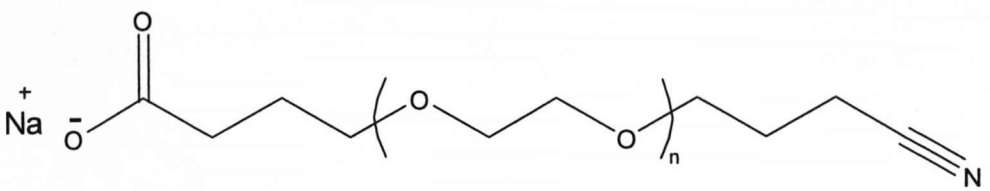
butyrate) and sodium PEG_{600} α,ω-(4-butyrate,4-butyronitrile) will be referred to as PEG-ba throughout the reminder of this thesis.



disodium PEG_{600}α,ω-di(4-butyrate)



sodium PEG_{600}(4-butyrate)



sodium PEG_{600}α,ω-(4-butyronitrile,4-butyrate)

Figure 4.1 schematic illustrating the structural formula of PEG-ba

Schematic showing the structural formula of disodium PEG_{600}di(4-butyrate), sodium PEG_{600}(4-butyrate) and sodium PEG_{600}(4-butyronitrile,4-butyrate). PEG-ba refers to a mixture of all three compounds utilised in the treatment of the colonic epithelial cell lines.

4.2 Results

HT-29 and AA/C1 colonic epithelial cells were treated with, i) sodium butyrate, ii) PEG-ba, iii) PEG_{600} or iv) butyronitrile. Sodium butyrate was used as a positive control, whilst PEG_{600} and butyronitrile were employed in order to assess any potential effect that they may have on the pattern of gene expression. Expression of IAP, p21, bak and bcl-x_L were determined at mRNA levels using either northern blot analysis or quantitative real time PCR, as appropriate. Protein expression for p21, bak and bcl-x_L were determined using western blot analysis with the specific antibodies. For alkaline phosphatase, due to the lack of a specific antibody to human alkaline phosphatase, the activity of IAP was determined enzymatically.

It is worth mentioning that the synthesis of PEG-ba proved to be a challenging task and took a large proportion of the time allocated to this study. In addition, the AA/C1 cell line is a very slow growing cell, this is in contrast to HT-29 cells which take approximately 1 week to reach confluency, allowing a greater number of passages to be utilised. Consequently, the number of experiments involving the AA/C1 cells had to be restricted to cover the essential work needed. For the above reasons the potential effect of butyronitrile on gene expression was only carried out using HT-29 cells

4.2.1 Regulation of intestinal Intestinal alkaline phosphatase and $p21^{WAF1/Cip1}$

IAP is a classical marker of differentiation, well-established to be up-regulated in response to butyrate treatment of HT-29 and other cells (Kruh *et al.*, 1995). P21 is a cell cycle inhibitor, the expression of which is responsive (up-regulated) to butyrate (Iacomino *et al.*, 2001; Litvak *et al.*, 1998). Previous work in the laboratory had shown that butyrate transport into the cell, via the MCT1, is required for butyrate to regulate IAP and p21. Therefore, it is expected that a membrane impermeable, non-transportable butyrate analogue would not be able to modulate the expression of IAP and p21. To test this, AA/C1 cells were grown to confluency, and were then either left untreated or treated with NaBut, PEG-ba, PEG_{600}, for 48 hours each at 5 mM concentration. In addition, HT-29 after reaching 80 % confluency were either left untreated or treated with 2 mM solutions of either NaBut, PEG-ba, PEG_{600} or butyronitrile for 24 hours. Different concentrations and incubation times selected were based on the previous work in the laboratory indicating the optimum conditions for butyrate effect using AA/C1 and HT-29 cells (Cuff *et al.*, 2005). Figure 4.2 shows that treatment of AA/C1 cells with NaBut, as expected, upregulated IAP mRNA expression and activity by 1.5 ± 0.22 and 1.2 ± 0.08 fold respectively. However there were no effect on IAP expression at both mRNA and activity levels when AA/C1 cells were treated with PEG-ba. In AA/C1 cells treated with PEG there was no statistically significant change in IAP mRNA nor activity.

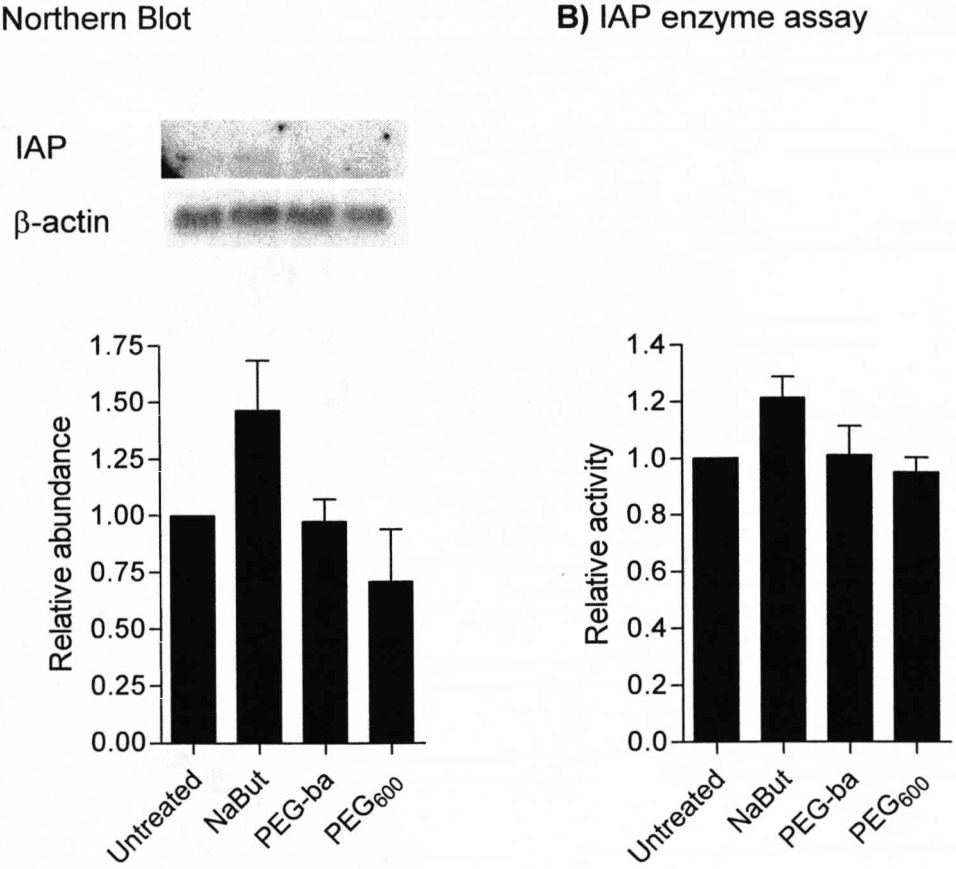


Figure 4.2 Expression of IAP in AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba and PEG_{600} for 48 hours, resupplementing after 24 hours. **A**, northern analysis showing IAP mRNA levels with densitometric analysis of abundance normalised to β-actin, as a histogram; **B**, histogram demonstrating IAP activity measured in the corresponding cell homogenate. Results are expressed, as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments.

Treatment of HT-29 cells with sodium butyrate resulted in 11 ± 1.35 and 9.2 ± 0.4 fold increase in IAP mRNA and activity (fig. 4.3). When HT-29 cells were treated with PEG-ba, a slight but not significant increase 1.4 ± 0.16 and 1.9 ± 0.41 fold were observed in mRNA and activity. There was also a slight, but not significant increase in IAP mRNA and activity in response of cells to exposure to PEG_{600}. However butyronitrile treatment of cells did not lead to any statistically significant change in IAP mRNA and activity.

It is worth mentioning that in response to butyrate treatment there was a 1.5 fold increase in IAP expression in AA/C1 cells, compared to 11 fold increase in IAP expression in HT-29 cells. As mentioned before HT-29 cell is an undifferentiated cell line which expresses negligible alkaline phosphatase. However in response to butyrate treatment it becomes differentiated and expresses high levels of the differentiation marker alkaline phosphatase. This is in contrast to AA/C1 cells, a differentiated phenotype, which expresses alkaline phosphatase; in response to butyrate treatment there is an additional increase in IAP but not as dramatic as seen in HT-29 cells.

As seen in figures 4.4 and 4.5 p21 expression is not also responsive to PEG-ba treatments. P21 expression is up-regulated 2 ± 0.7 and 1.6 ± 0.08 fold at mRNA and protein levels when AA/C1 cells are exposed to NaBut. However there is no enhancement in p21 in response to exposure of cells to either PEG-ba or PEG_{600}.

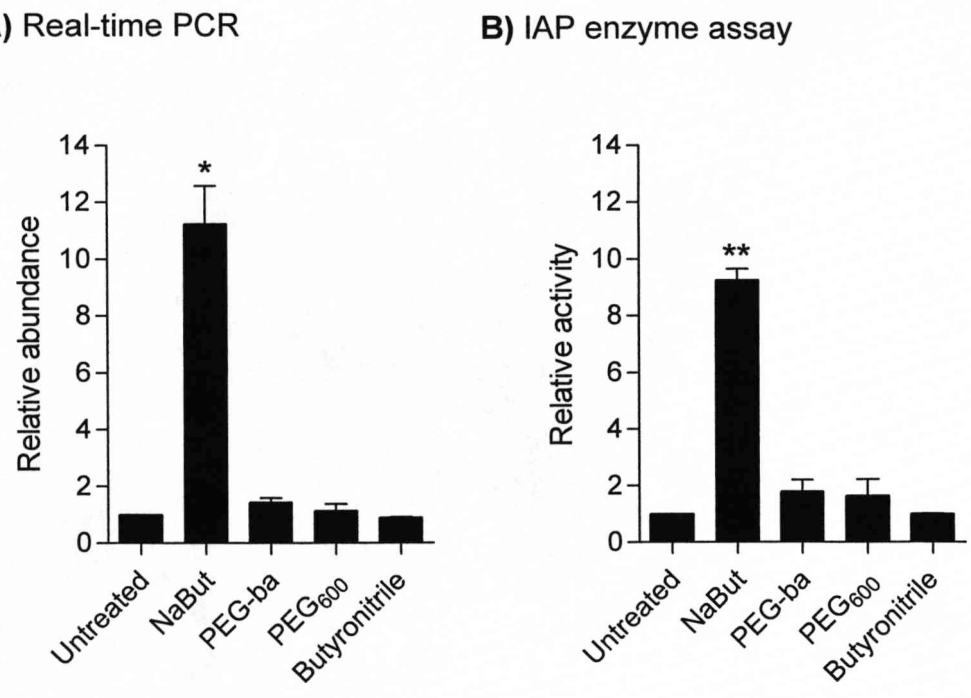


Figure 4.3 Expression of IAP in HT29 cells

HT-29 cells were grown to 70 % confluency and either left untreated or treated with 2 mM sodium butyrate, PEG-ba and PEG_{600} for 24 hours. **A**, real-time PCR analysis showing IAP mRNA levels with densitometric analysis of abundance normalised to β-actin shown as a histogram; **B**, Histogram demonstrating IAP activity measured in the corresponding cell homogenate. Results are expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05 and ** = p-value ≤ 0.005).

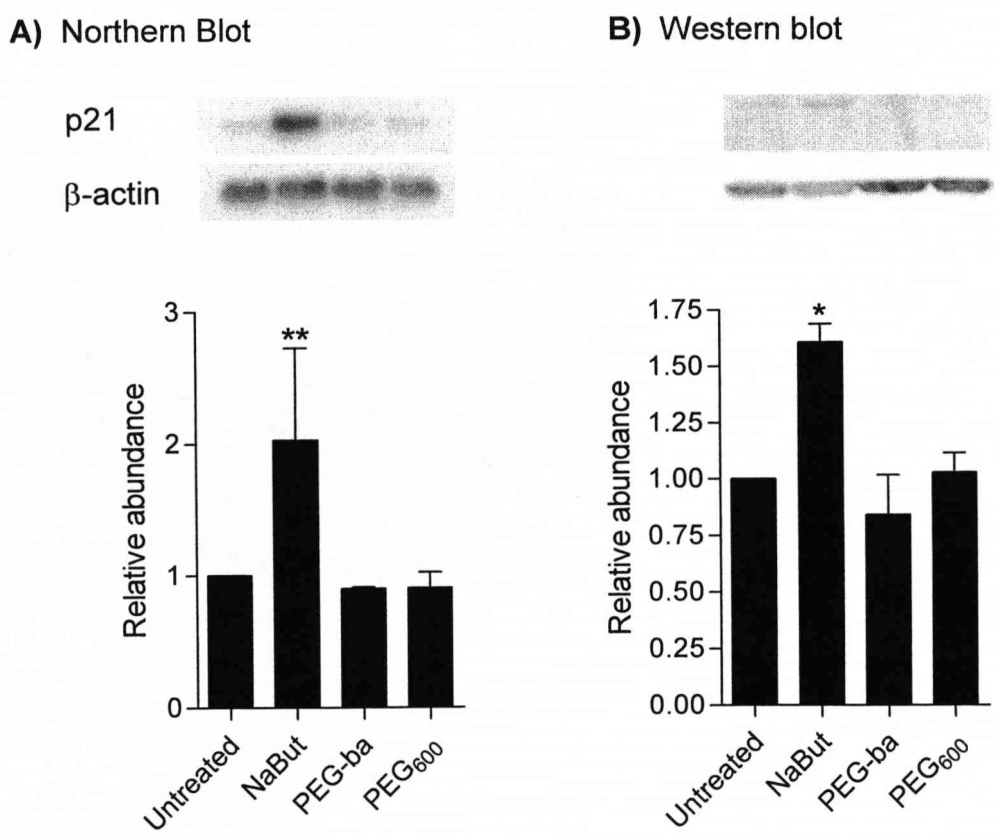


Figure 4.4 Expression of p21 in AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba and PEG_{600} for 48 hours, resupplementing after 24 hours. **A**, northern analysis showing p21 mRNA expression with corresponding densitometric analysis shown as a histogram; **B**, Western analysis showing p21 protein expression with corresponding densitometric analysis displayed as a histogram. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments. (* = p-value ≤ 0.05, ** = p-value ≤ 0.005).

Treatment of HT-29 cells with sodium butyrate resulted in a 1.6 ± 0.06 fold increase in p21 mRNA, as assessed by real-time PCR, and 1.5 ± 0.13 fold increase in p21 protein abundance (fig. 4.5). Treatment of HT-29 cells with PEG-ba and butyronitrile did not result in any statistically significant change in the expression of p21 mRNA nor protein. Treatment of PEG_{600} to HT-29 cells did not follow that seen in AA/C1 cells. Indeed, the relative abundance of p21 mRNA showed a 1.2 ± 0.04 fold decrease compared to untreated control. However there was a 1.4 ± 0.6 fold increase in protein abundance seen in PEG_{600} treated HT-29 cells compared to untreated control, resulting in conflicting data.

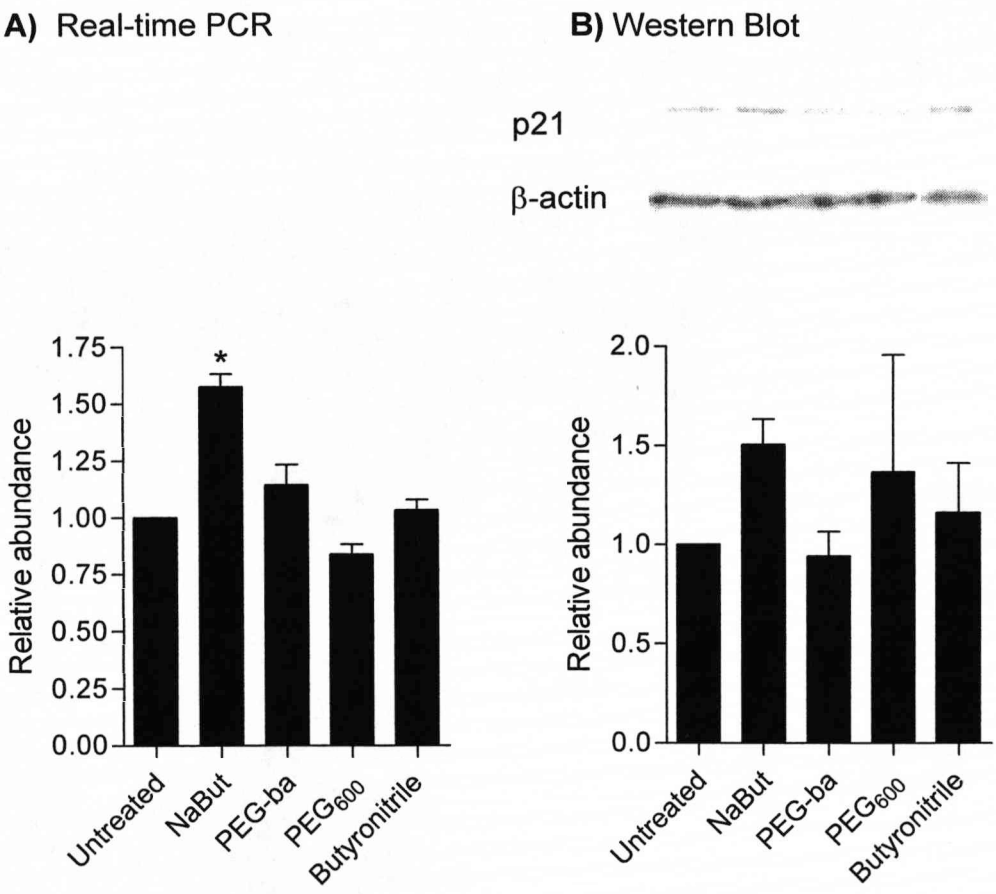


Figure 4.5 Expression of p21 in HT-29 cells

HT-29 cells were grown to 70 % confluency and either left untreated or treated with 2 mM sodium butyrate, PEG-ba and PEG_{600} for 24 hours. **A**, real-time PCR analysis showing p21 mRNA expression with the corresponding densiometric analysis shown as a histogram; **B**, Western analysis showing p21 protein expression with corresponding densiometric analysis shown as a histogram. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments. (* = p-value ≤ 0.05).

4.2.2 Regulation of Bcl-x_L and Bak

Previous work carried out in this laboratory has shown that knocking down the expression of the colonic butyrate transport, MCT1, had no effect on butyrate-regulation of *Bcl-x_L* and *Bak1* gene expression. *Bak1*, a pro-apoptotic gene is up-regulated by butyrate whilst *bcl-x_L*, an anti-apoptotic gene is down-regulated by butyrate (Cuff et al., 2005). AA/C1 and HT-29 cells were treated with PEG-ba and the effect on the expression of Bcl-x_L and Bak was investigated. Accordingly AA/C1 and HT-29 cells were treated with sodium butyrate, PEG-ba, PEG_{600} and butyronitrile (5 mM, AA/C1; 2 mM, HT-29) as mentioned above (section 4.2.1).

Treatment of AA/C1 cells with NaBut resulted in a 2 ± 0.16 fold reduction in the abundance of Bcl-x_L mRNA, examined by northern blotting, and 2.2 ± 0.03 fold reduction in the abundance of Bcl-x_L protein abundance, relative to an untreated control (fig. 4.6). The effect elicited by sodium butyrate was not seen when AA/C1 cells were treated with PEG-ba. Here, there was a 1.1 ± 0.1 fold and 1.4 ± 0.06 fold increase in the relative abundance of Bcl-x_L mRNA and protein levels respectively; the latter being statistically significant. Treatment of AA/C1 cells with 5 mM PEG_{600} had no effect on the abundance of Bcl-x_L mRNA; no statistically significant difference was seen in the relative abundance of Bcl-x_L protein compared to an untreated control (fig. 4.6).

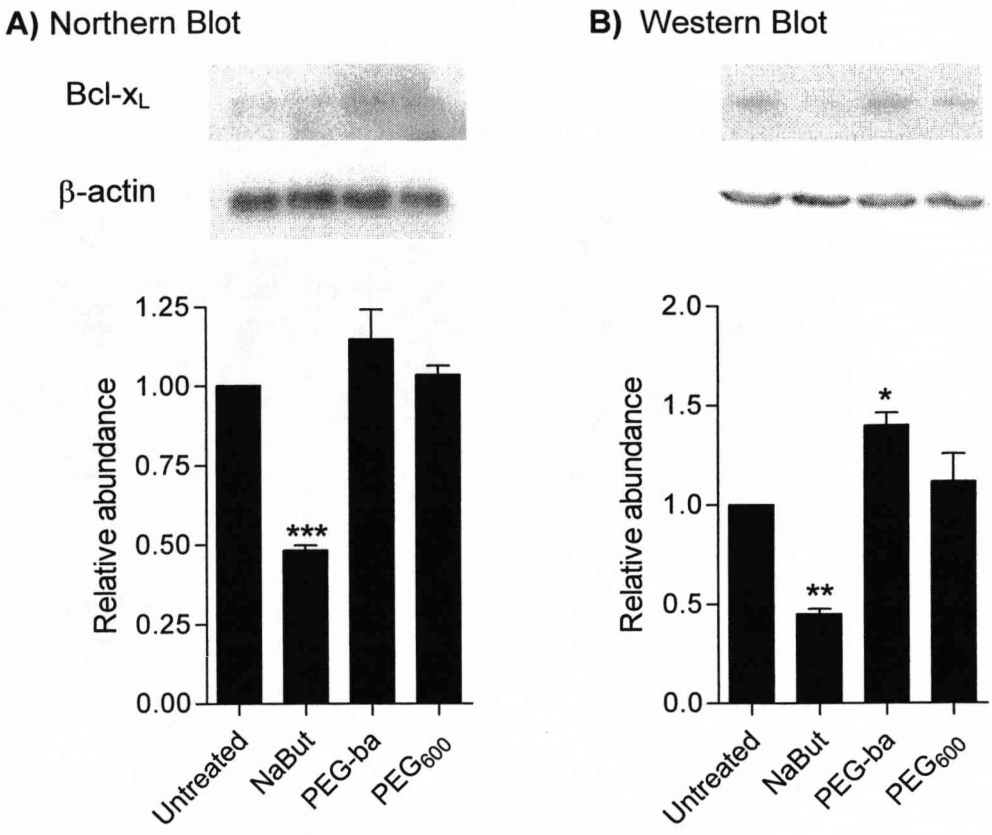


Figure 4.6 Expression of Bcl-x_L in AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba and PEG_{600} for 48 hours, resupplementing after 24 hours. **A**, northern analysis showing Bcl-x_L mRNA expression with the corresponding densiometric analysis show as a histogram; **B**, western analysis showing Bcl-x_L expression and the corresponding densiometric analysis. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05, ** = p-value ≤ 0.005 *** = p-value ≤ 0.001).

The pattern of expression for Bcl-x_L seen in AA/C1 cells was similar to that seen in HT-29 cells. Treatment of sodium butyrate to HT-29 cells resulted in a 1.45 ± 0.01 reduction in the abundance of Bcl-x_L mRNA and a 1.3 ± 0.05 fold reduction in the level of Bcl-x_L protein, compared to an untreated control (fig. 4.7). When HT-29 cells were treated with PEG-ba the opposite pattern of expression for butyrate treatment was seen; a 1.6 ± 0.09 fold and 1.7 ± 0.11 fold up regulation of Bcl-x_L mRNA and protein, respectively, relative to an untreated control that are both statistically significant. Treatment of HT-29 cells with PEG_{600} and butyronitrile did not result in any statistically significant change in the expression of Bcl-x_L mRNA or protein.

The pattern of expression for the pro-apoptotic gene Bak in AA/C1 and HT-29 cells treated with PEG-ba was very similar to that observed when cells were treated with NaBut. As shown in figure 4.8, in AA/C1 cells treated with sodium butyrate, there was a 1.8 ± 0.12 fold up regulation in Bak mRNA expression, as seen by northern blotting, and a 1.9 ± 0.06 fold up enhancement in the level of protein. When AA/C1 cells were treated with PEG-ba a 1.8 ± 0.11 fold and 2 ± 0.08 fold increase in Bak mRNA and protein respectively was observed; clearly demonstrating that PEG-ba can mimic the effects of butyrate. Treatment of PEG_{600} on AA/C1 cells resulted in a 1.3 ± 0.13 fold increase in the level of Bak mRNA. However, a smaller 1.1 ± 0.16 fold increase in the level of Bak protein was observed with PEG_{600} treatment; changes that are not statistically significant (fig. 4.8).

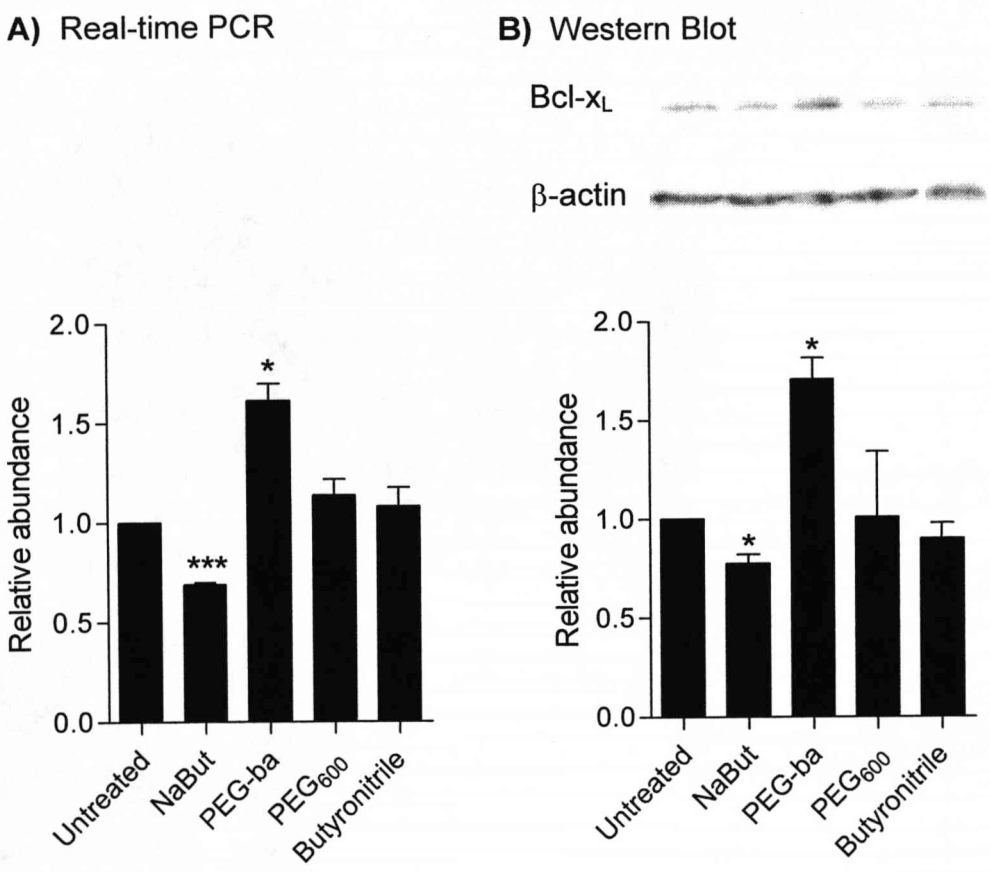


Figure 4.7 Expression of Bcl-x_L in HT-29 cells

HT-29 cells were grown to 70 % confluency and either left untreated or treated with 2 mM sodium butyrate, PEG-ba and PEG_{600} for 24 hours. **A**, real-time PCR analysis showing Bcl-x_L mRNA expression with the corresponding densiometric analysis shown as a histogram; **B**, western analysis showing Bcl-x_L protein expression and the corresponding denoiometric analysis. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05 and *** = p-value ≤ 0.001).

Treatment of HT-29 cells with sodium butyrate and PEG-ba showed a similar pattern of expression for Bak. As figure 4.9 shows, when HT-29 cells were treated with sodium butyrate, a 1.5 ± 0.05 fold increase in Bak mRNA and a 1.6 ± 0.07 fold increase in Bak protein were seen. Treatment of PEG-ba also increased the level of Bak mRNA and protein, albeit to a higher degree, with a 1.8 ± 0.05 and ± 0.12 fold increase in mRNA and protein respectively. Treatment of HT-29 cells with PEG_{600} and butyronitile had no statistically significant effect on the level of Bak mRNA and protein expression, (fig. 4.9).

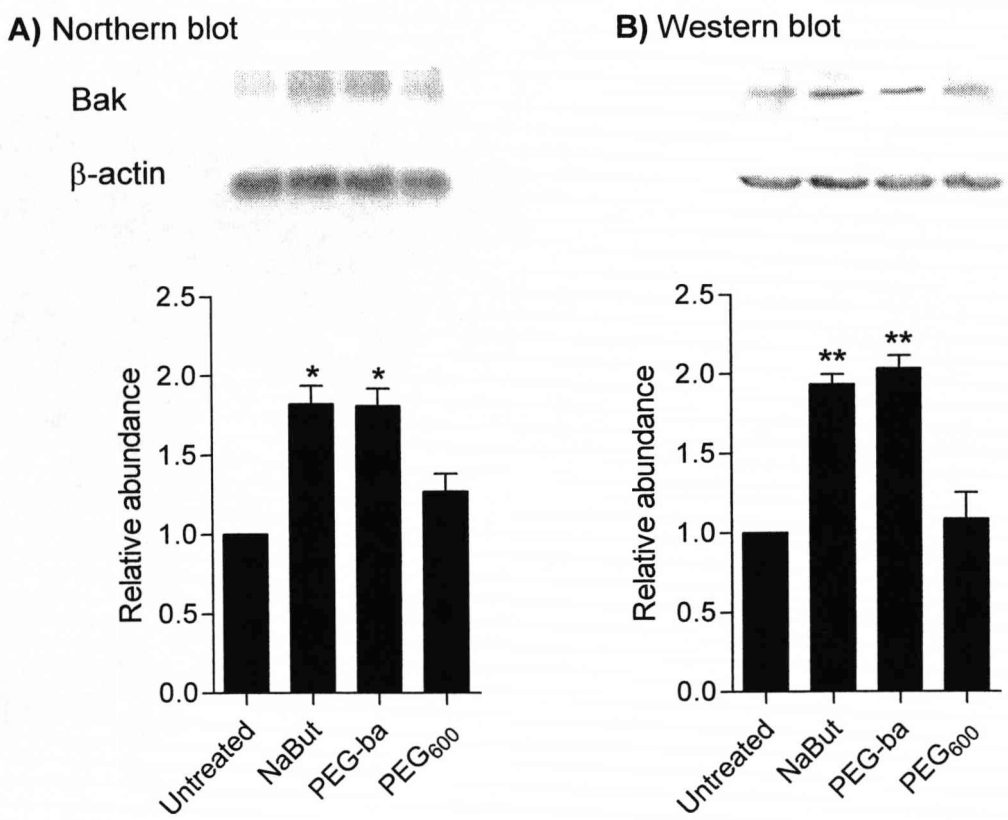


Figure 4.8 Expression of Bak in AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba and PEG_{600} for 48 hours, resupplemented after 24 hours. **A**, northern analysis showing Bak mRNA expression and the corresponding densiometric analysis shown as a histogram; **B**, western analysis showing Bak protein expression with the corresponding densiometric analysis shown as a histogram. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05, ** = p-value ≤ 0.005).

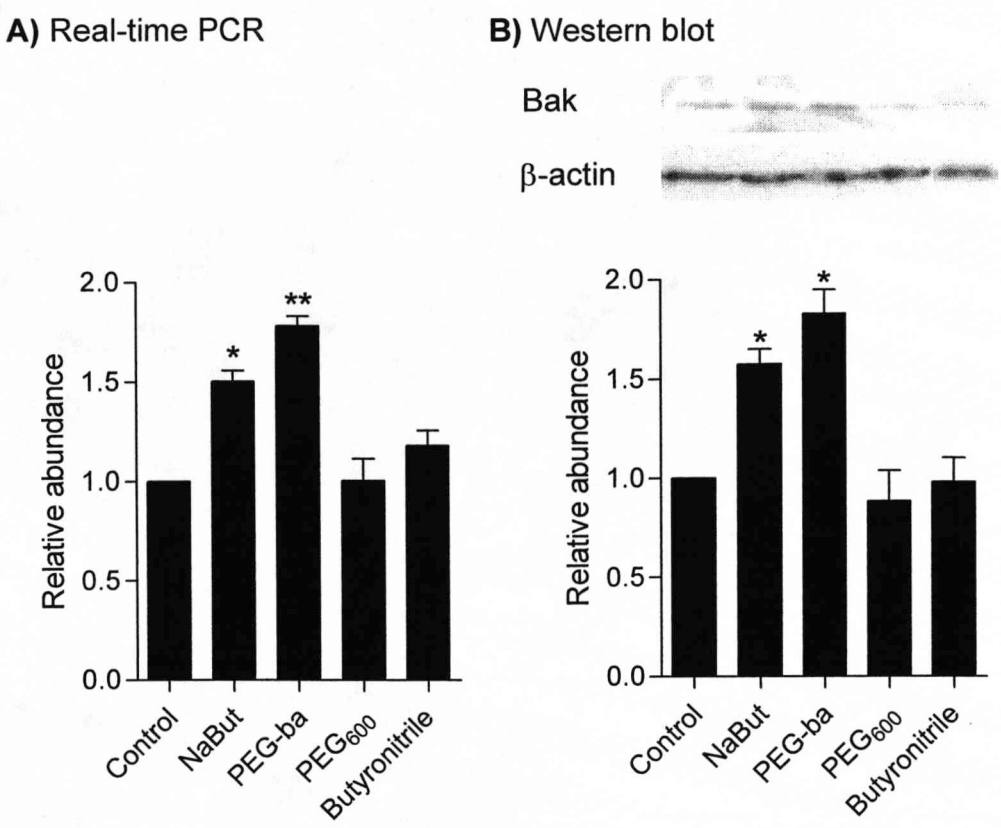


Figure 4.9 Expression of Bak in HT-29 cells

HT-29 cells were grown to 70 % confluency and either left untreated or treated with 2 mM sodium butyrate, PEG-ba and PEG_{600} for 24 hours. **A**, northern analysis showing Bak mRNA expression with the corresponding densiometric analysis displayed as a histogram; **B**, western analysis showing Bak protein expression with the corresponding densiometric analysis displayed as a histogram. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05 and ** = p-value ≤ 0.005).

4.2.3 Analysis of survivin, Bak and Bcl-x_L mRNA expression in AA/C1 cells by quantitative PCR

4.2.3.1 Analysis of Bak and Bcl-x_L mRNA expression

The mechanism by which butyrate regulates Bak and Bcl-x_L mRNA in AA/C1 cells was also investigated by real-time PCR. The results obtained were close to those obtained using HT-29 cells. As shown in figure 4.10, treatment of AA/C1 cells with sodium butyrate resulted in a 1.7 ± 0.04 fold enhancement in Bak mRNA expression. When AA/C1 cells were treated with PEG-ba a 2.1 ± 0.24 fold induction in Bak mRNA was seen. While PEG_{600} had no effect on the level of Bak mRNA expression. AA/C1 cells treated with sodium butyrate showed a 1.5 ± 0.02 fold down regulation of Bcl-x_L mRNA while treatment of PEG-ba resulted in a 1.5 ± 0.3 up regulation. When cells were treated with PEG_{600}, a smaller 1.2 ± 0.01 fold up regulation of Bcl-x_L mRNA was seen (fig. 4.10).

4.2.3.1 Analysis of survivin mRNA expression

Survivin is another anti-apoptotic gene known to be down-regulated by butyrate (Daly *et al.*, 2005). To investigate whether PEG-ba was able to modulate survivin expression, AA/C1 cells were treated with 5 mM of either butyrate or PEG-ba for 24 hours. Upon harvesting the cells, total RNA was isolated and cDNA was prepared. The relative expression of survivin was then assessed using real-time PCR. As shown in figure 5.10, butyrate down-regulated survivin 2.5 ± 0.02 fold while PEG-ba down-regulated survivin 4 ± 0.04 fold, compared to an untreated control.

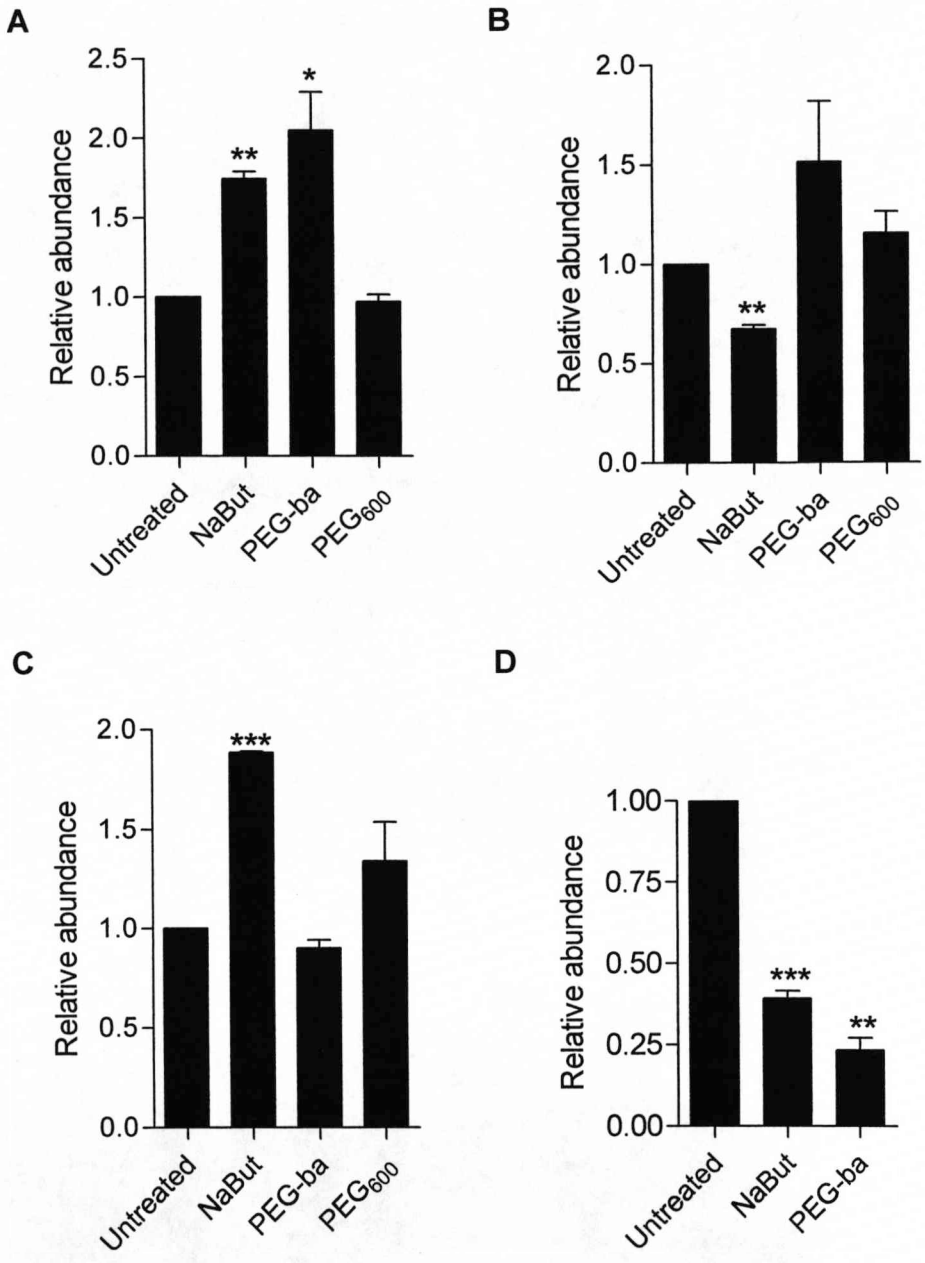


Figure 4.10 Real-time PCR analysis on AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba and PEG_{600} for 48 hours, resupplemented after 24 hours (Cells were treated for 24 hours for survivin, with no PEG_{600} control). Real-time PCR analysis was carried out for: **A**, Bak; **B**, Bcl-x_L; **C**, MCT1; **D**, Survivin. Results are expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (* = p-value ≤ 0.05, ** = p-value ≤ 0.005 *** = p-value ≤ 0.001).

4.2.4 Regulation of MCT1

The work described in this chapter clearly shows that PEG-ba, a membrane-impermeable, non-transportable butyrate analogue regulated the pro-apoptotic gene *bak* and the anti- apoptotic gene product survivin with the same pattern as butyrate. These finding supports our proposition that expression of these candidate genes controlling apoptosis may be regulated through "butyrate sensing". Our proposition is further supported by the findings that the regulation of IAP and p21 that was dependent on the entry of butyrate into the cell via MCT1 were not regulated by PEG-ba.

We next asked the question what is the pattern of regulation of MCT1 by butyrate? MCT1 is the colonic butyrate transporter, and it has been shown to be regulated by butyrate at both transcriptional and post-transcriptional levels (Cuff *et al.*, 2002). However, it is not known whether, for butyrate regulation of MCT1, there is a need for butyrate entry into the cell.

AA/C1 cells express MCT1 protein on the luminal membrane (Rizhaupt *et al.*, 1998a; Lambert *et al.*, 2002) and they have been used in studies assessing the regulation of MCT1 by butyrate (Cuff et al 2002). Accordingly, AA/C1 cells were grown to confluency and either left untreated or treated with NaBut or PEG-ba, 5 mM concentration, for 48 hours. Afterwards the cells were harvested and total RNA and cellular homogenates were prepared. Northern blot and real-time PCR were used to measure the abundance of MCT1 mRNA expression and western blot was employed to measure the MCT1 protein abundance.

Treatment of AA/C1 cells with NaBut resulted in a 1.6 ± 0.05 and 1.9 ± 0.01 fold increase in MCT1 mRNA abundance compared to control as measured by northern blotting and the real time PCR respectively (see fig. 4.10 and 4.11). There was a co-ordinated 2 ± 0.16 fold increase in MCT1 protein abundance

over the controls. No change was observed in either MCT1 mRNA or protein compared to controls when AA/C1 cells were treated with PEG-ba. In fact there was a reduction in MCT1 expression, at mRNA and protein levels in response to PEG-ba, indication that MCT1 regulation by butyrate requires butyrate entry into the cell.

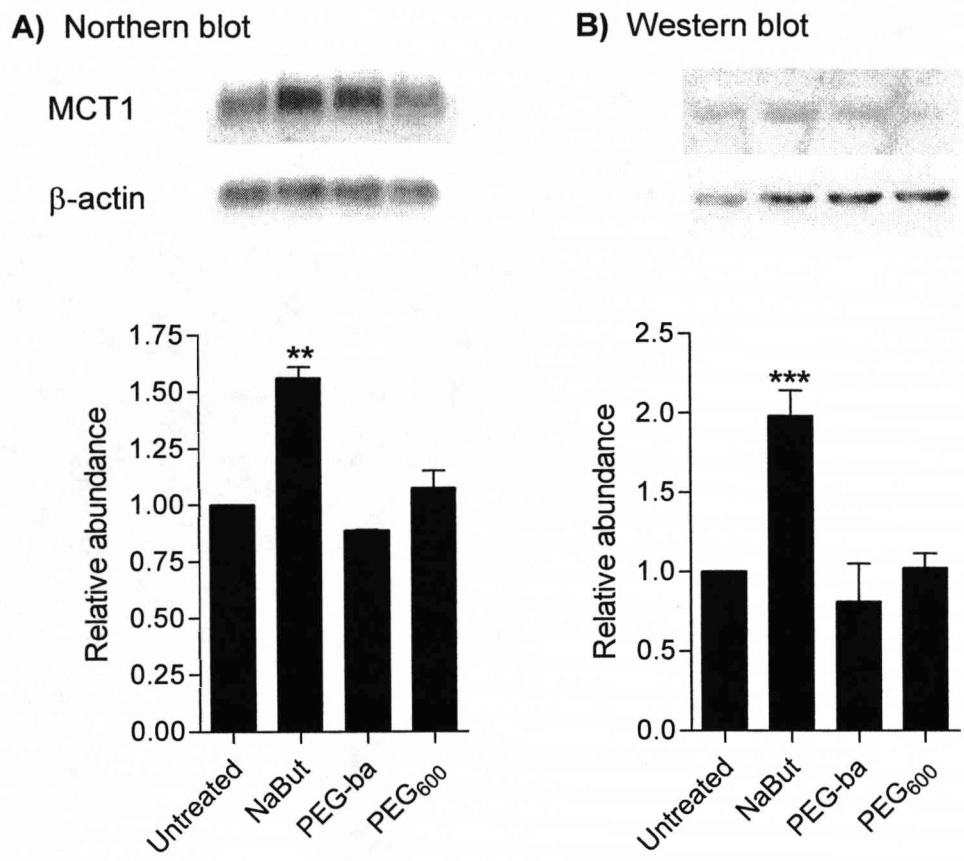


Figure 4.11 Expression of MCT1 in AA/C1 cells

AA/C1 cells were grown to 100 % confluency and either left untreated or treated with 5 mM sodium butyrate, PEG-ba or PEG_{600} for 48 hours, resupplemented after 24 hours. **A**, northern analysis showing MCT1 mRNA levels and the corresponding densiometric analysis, as a histogram; **B**, western analysis showing MCT1 protein levels with the corresponding densiometric analysis, as a histogram. Results are normalised to β-actin and expressed as abundance relative to untreated control; values represent mean ± S.E.M. of three separate cell treatments (** = p-value ≤ 0.05, *** = p-value ≤ 0.001).

4.3 Discussion

It has been shown by our laboratory and others that MCT1 is the protein responsible for the transport of butyrate across the colonic luminal membrane (Ritzhaupt *et al.*, 1998a; Stern *et al.*, 2000; Hadjiagapiou *et al.*, 2000) Furthermore we have shown that MCT1 is regulated by its substrate butyrate, and that knocking down MCT1 expression in several colonic cell lines results in the loss of butyrate's ability to regulate genes *AIP1* (gene product IAP), *CDKN1A* (CD1) and *CCDN1* (p21). In contrast the inhibition of MCT1 expression had no effect on butyrate's ability to up-regulate *bak1* (bak) and down-regulate *bcl-*x_L

We set out to synthesise a membrane-impermeable, non-transportable butyrate analogue to determine if extra-cellular butyrate can regulate the expression of *bak1* and *bcl-*x_L similar to that observed in response to butyrate. This objective was investigated because we hypothesised that for some genes to be regulated by butyrate, there is a need for butyrate entry into the cell, whilst for others butyrate may exert its effects via a "butyrate sensor" located on the external face of colonic luminal membrane. This hypothesis was strengthened not only by our previous findings but also by reports indicating the presence of G-protein coupled receptors, GPCR 41, and 43 that can act as butyrate sensors in the large intestine.

The membrane impermeable butyrate analogue was synthesised by coupling of the butyrate molecule, at the C4 position of butyrate, to either end of PEG_{600} (see figure 4.1 for the structural formula). An average molecular mass of 600 $gmol^{-1}$ for PEG was chosen as this is known to be impermeable to the intestinal plasma membrane (Lloyd, 1998) and yet it is soluble. PEG-ba was fully characterised by ^{1}H and ^{13}C NMR and mass spectrometry.

Human colonic cell lines AA/C1 (derived from non-tumourigenic Adenoma) and HT-29 (originated from an adenocarcinoma) were utilised to assess the effect of PEG-ba on gene expression. The expression of IAP, p21, bak and bcl-x_L after treatment with PEG-ba was investigated in both cell lines; the expression of MCT1 and survivin after PEG-ba treatment was also investigated in AA/C1 and HT-29 cell lines respectively. The change in the expression of each gene in response to PEG-ba treatment was determined relative to the untreated control cells. The levels of expression were then compared to that observed with cells treated with butyrate (positive control) and PEG_{600} and butyronitrile (negative controls).

The results indicated that PEG-ba had no regulatory effect on the expression levels of *CDKN1A* and *ALP1* in both AA/C1 and HT-29 cells compared to the effects seen by butyrate treatment. We had shown previously that butyrate was not able to modulate the expression of the above genes when MCT1 expression was silenced, using siRNA, in AA/C1 and HT-29 cells (Cuff et al 2005).

PEG-ba was able to modulate the expression of bak at the level of mRNA and protein, and survivin at the mRNA level (due to the lack of antibody to survivin it was not possible to determine protein expression). In AA/C1 a 1.8 ± 0.11 and 2 ± 0.08 fold enhancement in mRNA and protein were observed in response to PEG-ba treatments; values identical to the effect seen by butyrate. PEG-ba treatment of HT-29 cells resulted in a 1.8 fold ± 0.11 and 0.16 increase in bak mRNA and protein. This increase was slightly higher to that observed for bak by butyrate, which resulted in a 1.5 ± 0.05 and 1.6 ± 0.07 fold increases in bak mRNA and protein respectively. In contrast PEG-ba down regulated survivin mRNA 4 ± 0.04 fold in AA/C1 cells compared to a 2.5 ± 0.02 fold reduction by NaBut. These data collectively indicate that p21 and IAP are only

up-regulated in response to butyrate, and are not responsive to PEG-ba. In contrast, both butyrate and its membrane impermeable analogue PEG-ba regulate bak and survivin to an extent (bak expression is up-regulated by both butyrate and PEG-ba, whilst survivin is down regulated by both compounds); supporting the notion that for the latter genes, butyrate may exert its effect through a plasma membrane butyrate sensing molecule.

There were some changes in gene expression in response to PEG_{600} treatments, more pronounced in HT-29 cells than AA/C1 cells, but by no means were the changes as dramatic as seen with PEG-ba. There are reports of PEG inducing differentiation in HT-29 cells in the literature. Laboisse *et al.*, 1988 reported that treatment of PEG_{1000} on HT-29 cells brought about a differentiated subpopulation of cells with characteristics of mucous secreting goblet cells and endocytotic cells. Both subpopulations also showed reduced tumourogenicity than the parental HT-29 cell line (Laboisse *et al.*, 1988). PEG has also been found to induce apoptosis in HT-29 cells (Roy *et al.*, 2001). Parnaud *et al.*, 2001 reported that treatment of PEG_{8000} on HT-29 cells reduced proliferation through reducing the number of cells in S phase and increasing the number of cells in G_0/G_1. The authors noted that the treatment of PEG_{8000} increased the number of floating cells within the medium. Upon further investigation, blebbing – a characteristic of apoptosis – was seen, and no necrotic cells were found (Parnaud *et al.*, 2001).

It is worth noting that the up regulation in the abundance of p21 and IAP induced by PEG_{600} is not seen by PEG-ba. Furthermore, the level to which PEG_{600} up regulates bak, a 1.3 fold induction in AA/C1 cells, is not statistically significant. Nor is it to the same level induced by PEG-ba that gave a 1.8 fold increase in bak mRNA in AA/C1 cells; an increase that was statistically significant. The size of PEG used seems to be crucial for inducing apoptosis

with PEGs of around 8000 Da seeming to be the optimum size for inhibiting biomarkers for colon cancer (Roy *et al.*, 2001); this being well above the size of PEG used in this study.

Butyrate has been shown previously to regulate the expression of its transporter, MCT1. We sought to identify whether butyrate sensing or transport was responsible for MCT1 upregulation by butyrate. Treatment of AA/C1 with PEG-ba failed to modulate the expression of MCT1 indicating that butyrate entry is required for butyrate regulate of MCT1 expression.The data presented in this chapter clearly supports our previous results (Cuff *et al.*, 2005) indicating MCT1 expression and addressing its effects on butyrates ability to modulate the expression of key target genes. The combined data collectively support the suggestion for the presence of an extra-cellular "butyrate sensor" that senses luminal butyrate. The sensor may activate an intra-cellular pathway resulting in the downstream regulation of Bak, survivin and Bcl-x_L.

There is evidence in the literature concerning nutrient sensing by the intestinal epithelium and the downstream pathways. Long-chain fatty acids, amino acids and sugars are sensed in the intestine while (Raybould, 1999) amino acid transporters and the sugar transporter, SGLT1, are regulated via extra-cellular sensors (Dyer et al., 2003; Hyde *et al.*, 2003). The intestinal calcium-sensing receptor is activated by a multitude of divalent ions as well as L-amino acids, small peptides and polyamines (Riccardi & Maldonado-Perez, 2005) and is responsible for fluid secretion in the colon (Geibel *et al.*, 2006).

Suitable candidates for the butyrate receptor are the G-protein coupled receptors, GPR41 and GPR43, which are activated by short chain carboxylic anions in a dose-dependent and dose-specific manner (Brown *et al.*, 2003). Moreover, GPR41 and GRP43 have been found to induce various biological

pathways upon activation by SCFAs (Kimura *et al.*, 2001; Xiong *et al.*, 2004). On going work in our laboratory has identified that the G-protein coupled receptors GPR41 and GPR43 are present in AA/C1 and HT-29 cell lines and on the luminal membrane of human colon. As GPR41 and GPR43 have been shown to be activated by butyrate, it is feasible that either GPR41 or GPR43 are responsible for the regulation of bak and survivin in AA/C1 and HT-29 cells by butyrate.

In this study, PEG-ba was statistically unable to regulate the expression of IAP and p21; but was able to regulate the expression of Bak, to the same magnitude as NaBut treatment. The pattern of expression for p21, IAP and Bak in AA/C1 and HT-29 cells, after PEG-ba treatment agrees with the previous data obtained from silencing MCT1 using siRNA i.e. silencing of MCT1 prevented butyrate's ability to modulate the expression of p21 and IAP, but not Bak (Cuff *et al.*, 2005). However, the up-regulation of Bcl-x_L by PEG-ba does not conform to the expected pattern of expression determined after silencing of MCT1 expression.

Silencing of MCT1 had no effect on butyrate's ability to modulate Bcl-x_L, showing a 2 fold reduction in Bcl-x_L expression, post butyrate treatment (Cuff et al., 2005). Treatment of AA/C1 and HT-29 cells by NaBut resulted in a 2 fold decrease in Bcl-x_L expression compared to a 2 fold increase in Bcl-x_L expression after PEG-ba treatment; both statistically significant. The data presented here shows that although PEG-ba can mimic butyrate in modulating bak expression in AA/C1 and HT-29 cells and survivin in AA/C1 cells, it is not entirely the case for bcl-x_L.

Butyrate increases apoptosis by up regulating pro-apoptotic genes, such as *Bak1* and down regulating anti-apoptotic genes, such as *BIRC5* (survivin) and

Bcl-x$_L$. The up regulation of Bcl-x_L seen here by PEG-ba does not fit with this pattern of expression and requires further investigation.

If the induction of Bak by PEG-ba does involve the sensing of PEG-ba by an extra-cellular nutrient sensor, then it is possible for Bak, survivin and Bcl-x_L to be regulated by different sensors. If only one sensor was responsible for the induction of Bak and the inhibition of survivin and Bcl-x_L then one would expect PEG-ba to mimic butyrate's effect in modulating these three genes. If there are multiple sensors involved in the sensing of butyrate, then these may be differentially expressed with different affinities for butyrate. PEG-ba may not fulfil the requirement of butyrate to regulate the expression of Bcl-x_L. One way to help clarify this issue would be to knock down GPR41 and GPR43 using siRNA, and determine if GPR41 and 43, or both, were responsible for sensing butyrate in the colon and if Bak, survivin and Bcl-x_L expression was regulated by these GPCRs.

The 2 fold increase in Bcl-x_L expression following PEG-ba treatment, may also be due to non-specific interactions between the PEG-ba and the putative 'butyrate sensor'. Co-treatment of NaBut and PEG-ba would help to determine whether the increased expression of Bcl-x_L following PEG-ba treatment is a true result, or merely an anomaly. If co-treatment of NaBut and PEG-ba resulted in the down-regulation of Bcl-x_L (i.e. mirroring NaBut treatment), then the PEG-ba-induced up-regulation of Bcl-x_L would more likely be an anomalous result. Whereas, if no change in Bcl-x_L expression was seen (i.e. NaBut-induced down-regulation being cancelled out by PEG-ba-induced up-regulation), then the up-regulation of Bcl-x_L is probably a true result.

In summary, the work presented in this chapter demonstrates that the membrane-impermeable butyrate analogue is capable of mimicking butyrates ability to modulate butyrate-responsive genes such as *bak1* and *BIRC5*.

Furthermore, the data presented here supports the notion of the presence of an extra-cellular butyrate sensor(s) present on the plasma membrane of the two colonic cell lines, AA/C1 and HT-29. The potential mechanism could be that the sensor senses media butyrate activating a downstream pathway resulting in the regulation of the two apoptotic genes, *bak1* and *BIRC5*.

CHAPTER 5

Exchange of butyrate/heptafluorobutyrate (HFB) across porcine colonic luminal membrane vesicles; treatment of human colonic epithelial cell lines with HFB

5.1 Introduction

As discussed in previous chapters, the experimental data have indicated that the ability of butyrate to modulate the expression of candidate genes p21 and IAP (involved in the control of proliferation and differentiation) is dependent on its transport into the cell. Butyrate serves as the principal source of energy for colonocytes (Roediger, 1980), a role for which cellular entry clearly is vital. It is feasible, therefore that the requirement for butyrate entry may reflect a metabolic dependency of its actions on proliferation and differentiation that is not required for modulation of apoptosis. Heerdt *at al.*, (1994) investigated the effect of a non-metabolisable butyrate analogue heptafluorobutyrate, (HFB) on butyrate mediated differentiation and apoptosis in HT-29 and SW620 colon adenocarcinoma cell lines. They did not detect any modulations in apoptosis or differentiation of these cells by HFB. They concluded that butyrate-induced apoptosis and differentiation of HT-29 and SW20 colonic cell lines may be dependent on butyrate metabolism (Heerdt *et al.*, 1994).

Although Heerdt *et al.*, (1994) had used HFB as a nonmetabolisable butyrate analogue; there was no indication that HFB is transported into the cell. Fluorine has been used in a wide variety of metabolite analogues. Fluorine carbon bond energies are one of the highest found in natural products and known to be broken enzymatically (Goldman, 1969). The transport of HFB into the cell and the potential involvement of HFB as a non-metabolisable butyrate analogue regulating gene expression were investigated in the present study.

In order to investigate if HFB is transported across the colonic luminal membrane, we used porcine colonic tissue due to its functional similarity to human (Sullivan *et al.*, 2001). Colonic mucosal scrapings were used to prepare

colonic luminal membrane vesicles. The use of membrane vesicles to study the transport of nutrients and ions across plasma membranes is a well established technique that has many advantages over whole cell and tissue experiments. The transport of nutrients being investigated are not subjected to metabolism, and the extra and intra cellular composition can easily be manipulated to suit the experimental design (Murer *et al.*, 1984). There are disadvantages to the use of membrane vesicles. Routine preparations are never free of contaminating membranes; during transport studies, the solute under investigation can bind to the membrane giving false positive results; and the membranes may become leaky. Control experiments are therefore carried out to assess and minimise the contribution of other factors (Murer & Kinne, 1980). Purified colonic LMVs were prepared using cation precipitation and differential centrifugation technique as reported by Ritzhaupt *et al.*, (1998a) and as discussed in the Methods.

Once isolated the colonic LMV are characterised in terms of purity and potential contamination by other membranes such as the basolateral and organelle membranes. Characterisation can be carried out by assessing the enrichment of marker proteins for the luminal and basolateral membranes using western blotting. Enzyme assays against marker enzymes for luminal, basolateral and organelle membranes are also utilised to assess any potential contamination by these membranes.

The purpose of work carried out in this chapter was not to characterise butyrate uptake, as this has been reported previously; the work carried out in this chapter set out to determine if HFB is transported into colonic LMVs, and if HFB could mimic the effects of butyrate on butyrate-responsive genes.

5.2 Results

5.2.1 Characterisation of colonic luminal membrane vesicles

Characterisation of colonic LMVs was carried out by immunoblotting for marker proteins characteristic of the luminal and basolateral membranes of colonocytes.

5.2.1.1 immunodection of markers of colonic luminal membranes

Colonocytes have characteristic microvilli present on the luminal face of the epithelial cell. Villin is a 95 kDa protein that is associated with α-actin in the microvilli of epithelial cells, and is a reliable marker of the luminal membrane of colonoctyes (Robine *et al.*, 1985). MCT1 is also another indicator of the luminal membrane of porcine and human colon (Ritzhaupt *et al.*, 1998b) and has been shown to reside on the luminal membrane of the human colon by immunohistochemistry (Lambert *et al.*, 2002).

In order to assess the abundance of luminal marker proteins, 20 μg colonic LMV and homogenate were separated on 8 % SDS-PAGE gels and transferred to PVDF membrane before blotting with antibodies to villin and MCT1. As shown in figure 5.1 villin was enriched in the colonic LMV fraction by 2.2 ± 0.33 fold, compared to homogenate. In addition MCT1 showed a 4 ± 1.5 fold enrichment in the colonic LMV fraction, compared to the homogenate (fig. 3.1).

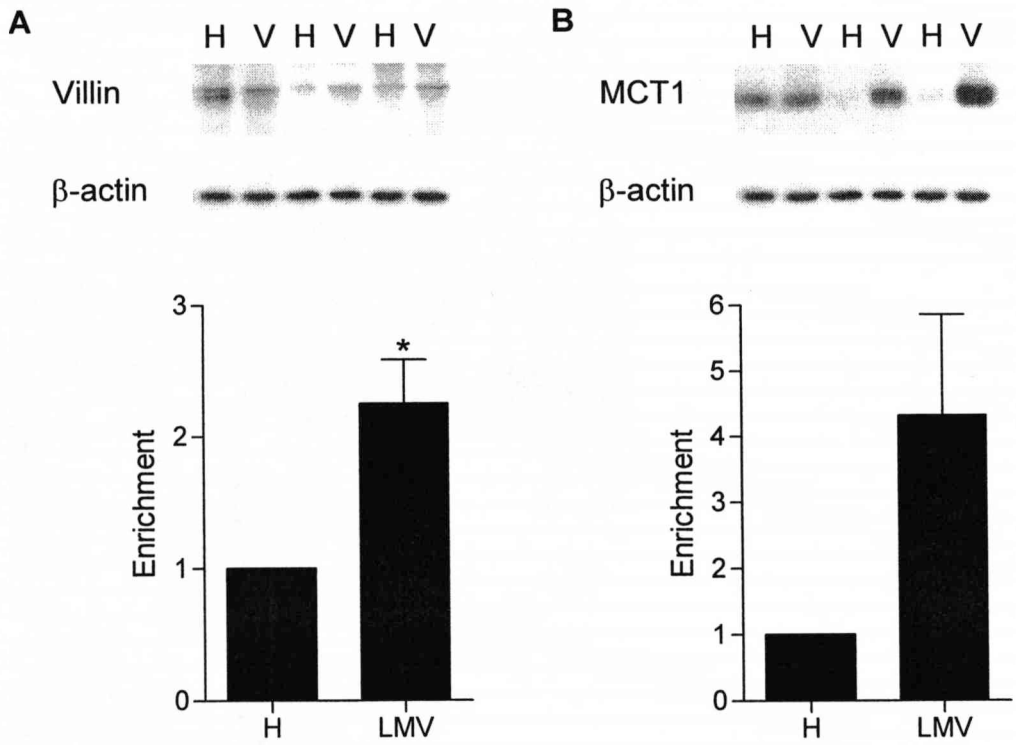


Figure 5.1 Enrichment of colonic luminal membrane markers in porcine colonic LMVs

Porcine colonic LMV (20 μg) were separated on SDS-PAGE gels, transferred to PVDF membrane and immunoblotted for luminal membrane marker proteins villin and MCT1. H = crude cellular homogenate, V = purified colonic LMVs. **A**, western blot for villin showing two fold enrichment in colonic LMVs compared to homogenate. **B**, western blot showing a four fold enrichment of MCT1 in colonic LMV compared to homogenate. Results are expressed as mean ± S.E.M. of 3 separate LMV preparations (* = p-value ≤ 0.05).

3.2.1.2 Immunodetection of markers of colonic basolateral membranes

HLA-1 is a member of the human major histocompatibility (MHC) antigen family. Class I and II MHC antigens are known to play a central role in the response to antigens in the body (Gorvel *et al.*, 1984). HLA class I (HLA-1) has been localised to the basolateral membrane of human jejunal enterocytes (Gorvel *et al.*, 1984) and porcine RLA class I has been localised to the basolateral membrane in pig colonocytes (Pinches *et al.*, 1993). To assess any potential contamination with basolateral membranes in the colonic LMV the abundance of HLA-1 was measured using western blotting. As shown in figure 3.2, HLA-1 displayed a 3.33 ± 0.12 fold disenrichment in the LMV fraction compared to the homogenate, indicating that the colonic LMVs had hardly any basolateral membrane contamination.

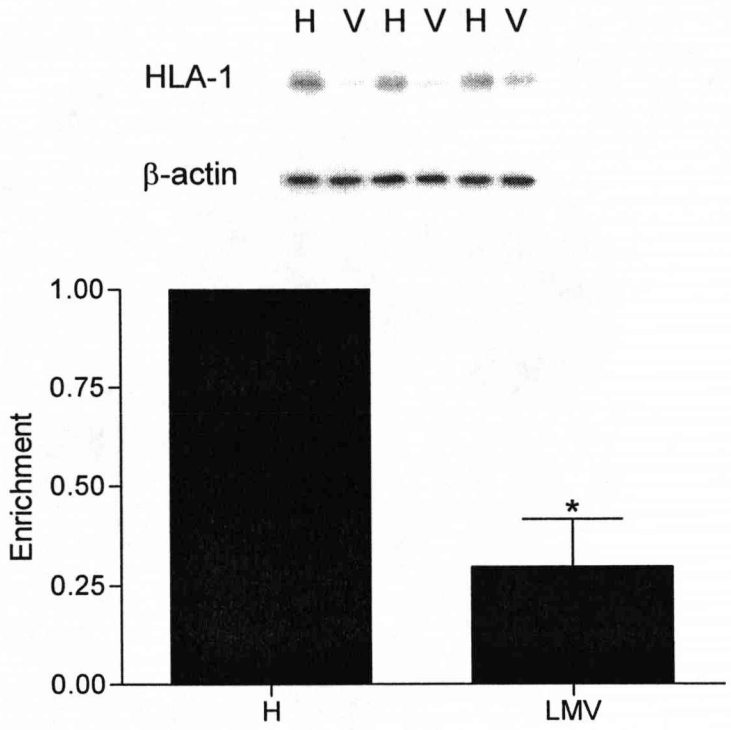


Figure 5.2 Enrichment of HLA-1 in porcine colonic LMVs

Porcine colonic LMV (20 μg) were loaded on SDS-PAGE gels, transferred to PVDF membrane and immunoblotted for HLA-1, a marker of the basolateral membrane, showing 4 fold disenrichment in colonic LMV compared to homogenate. H = crude cellular homogenate, V = purified colonic LMVs. Results are expressed as mean ± S.E.M. of 3 separate LMV preparations.

5.2.1.3 Assessment of LMV purity by enzyme assays

Cysteine-sensitive alkaline phosphatase (AP) is a membrane-bound glycoprotein with an approximate molecular mass of 66 kDa. It hydrolyses a wide variety of monophosphate esters at an alkaline pH (Malo *et al.*, 2004). Cysteine-sensitive AP is a classical luminal membrane marker and its abundance was assessed using a cysteine-sensitive AP enzyme assay. Accordingly, the activity of cysteine-sensitive AP was assessed in the homogenate and LMV fraction to calculate the enrichment and recovery of this luminal membrane marker. As shown in table 5.1 cysteine-sensitive AP was enriched 7 fold in the colonic LMV fraction and had a recovery of 23 %. The activity of the luminal membrane marker, H^+/K^+-ATPase, was also assessed using a H^+/K^+-ATPase enzyme assay. As shown in table 5.1 the activity of H^+/K^+-ATPase was enriched 5 fold with a recovery of 15.9 %.

Contamination of colonic LMVs with organelle membranes was assessed by assaying for marker enzymes for the mitochondria, endoplasmic reticulum (ER) and Golgi apparatus. The enzymes succinate dehydrogenase, a marker for the mitochondria; tris-resistant-α-glucosidase, a marker for the ER; and α-mannosidase, a marker for the Golgi apparatus were assessed in the LMV fraction to assess any potential contamination by these membranes. The enrichment and recoveries of marker enzymes from organelle membranes were low. Tris-resistant-α-glucosidase was enriched 1.8 fold and had a recovery of 6.7 %. α-mannosidase and succinate dehydrogenase had small enrichments of 1.6 and 2 fold respecitively; with recoveries of 4.5 and 5.7 % respectively (see table 5.1).

Table 5.1 Activities of marker enzymes in colonic LMVs

Enzyme	Enrichment[1]	Recovery[2] / %	Specific activity nmoles. min.$^{-1}$ (mg protein)$^{-1}$	n
Cysteine-sensitve alkaline phosphotase	6.99 ± 1.02	23.25 ± 3.59	7.39 ± 1.19	3
H^+/K^+ - ATPase	5.06 ± 0.848	15.92 ± 1.253	4.73 ± 2.771	3
tris-resistant-α-glucosidase	1.82 ± 0.51	6.66 ± 3.68	1.03 ± 0.11	3
α-monnosidase	1.55 ± 0.35	4.47 ± 1.25	1.74 ± 0.70	3
succinate dehydrogenase	1.97 ± 0.48	5.73 ± 1.13	1.57 ± 0.71	3

Membrane vesicles were isolated and enzyme assays were performed as described in Methods sections 2.4.2 and 2.5 respectively. The assays were performed in duplicate. Activities are expressed as mean ± S.E.M. of 3 separate vesicle preparations.

[1]Enrichment refers to the ratio of the specific activity of the enzyme in the LMV fraction over the specific activity of the enzyme in the homogenate fraction.

[2]Recovery refers to the total activity of the enzyme in the LMV fraction as a percentage of the total activity present in the homogenate.

5.2.3 Transport of sodium [U-^{14}C]-butyrate

In order to assess the transport of the non-metabolisable butyrate analogue, HFB, we first set out to reproduce the typical butyrate uptake into porcine colonic LMV carried out previously in this laboratory (Ritzhaupt *et al.*, 1998a).

5.2.3.1 Butyrate/$^-$OH exchange

Butyrate/proton co-transport into LMV was investigated. Accordingly LMV were preloaded with 300 mM mannitol, 20 mM Hepes/tris pH 7.5 and 0.1 mM $MgSO_4$. Butyrate uptake was performed at 37 °C in a buffer consisting of 100 mM mannitol, 100 mM Na gluconate, 1 mM butyrate with tracer amounts of [U-^{14}C]-butyrate, 0.1 mM $MgSO_4$ and either 20 mM Hepes/tris pH 7.5 or 20 mM Mes/tris pH 5.5 for 5 s. As shown in figure 5.3 the rate of butyrate uptake at the extravesicular pH (pH_{out}) of 7.5 was 17.31 ± 5.11 pmoles/5 s/mg protein compared to 382.4 ± 42.24 pmoles/5 s/mg protein when pH_{out} = 5.5. The rates for butyrate/proton co-transport (butyrate/hydroxyl exchange) in porcine colonic LMV were comparative to the rates determined previously.

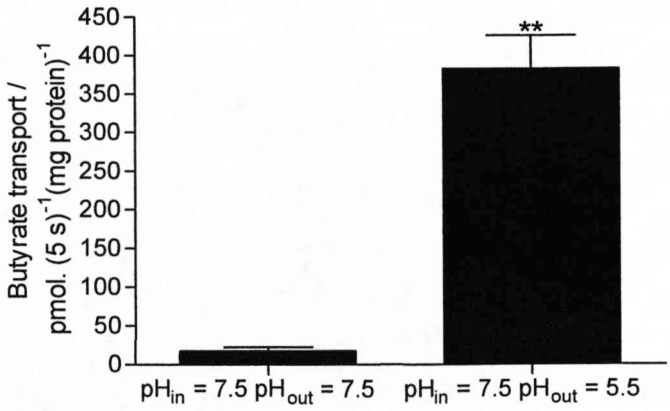


Figure 5.3 Uptake of Butyrate into Porcine colonic LMV

Vesicles were loaded with 300 mM mannitol, 20 mM Hepes/tris pH 7.5 and 0.1 mM $MgSO_4$. LMV (50 μg protein per assay) were incubated in 100 mM mannitol, 100 mM sodium gluconate, 1 mM butyrate, 0.1 mM $MgSO_4$ and either 20 mM Hepes/tris pH 7.5 or 20 mM Mes/tris pH 5.5. Uptake was measured as described in section 2.8. (pH_{in} = intravesicular pH, pH_{out} = extravesicular pH). Rates are expressed as mean ± S.E.M. for three separate LMV preparations (** = p-value ≤ 0.05).

5.2.3.2 Effect of intravesicular anion

We next set out to reproduce the uptake of butyrate into the LMV in exchange for intra-vesicle butyrate. Butyrate/butyrate exchange was determined using the same method as above with colonic LMV preloaded with 100 mM mannitol and 100 mM NaBut. Figure 5.4a shows the rates of butyrate uptake into colonic LMVs in exchange for butyrate, with a differing extravesicular pH of 7.5 and 5.5. The rate of uptake into colonic LMVs loaded with butyrate was 436.7 ± 48.69 pmoles/5 s/mg protein at pH_{out} = 7.5 and 1239 ± 131.6 pmoles/5 s/mg protein at pH_{out} = 5.5.

We next set out to determine the rate of HFB transport into colonic LMVs. As no ^{14}C radiolabelled HFB was commercially available, no direct tracer-labelled transport using a ^{14}C-labelled HFB were possible. HFB transport was therefore determined using trans-stimulation of HFB in exchange for transport of radio-labelled butyrate into the vesicles. Accordingly, LMV were preloaded with either 300 mM mannitol or 100 mM mannitol and 100 mM NaBut or NaHFB. Butyrate uptake was performed in either extravesicular Hepes/tris buffer pH 7.5 or Mes/tris buffer pH 5.5. As shown in figure 5.4a the rate of butyrate uptake into colonic LMVs in exchange for HFB was 17.51 ± 3.0 and 247.9 ± 44.55 pmoles/5 s/mg protein at pH_{out} 7.5 and pH_{out} 5.5 respectively; a rate much smaller than butyrate/butyrate exchange and a smaller rate than butyrate/hydroxyl exchange (17.31 ± 5.11 and 382.4 ± 42.24 pmoles/5 s/mg protein at pH_{out} = 7.5 and pH_{out} = 5.5 respectively).

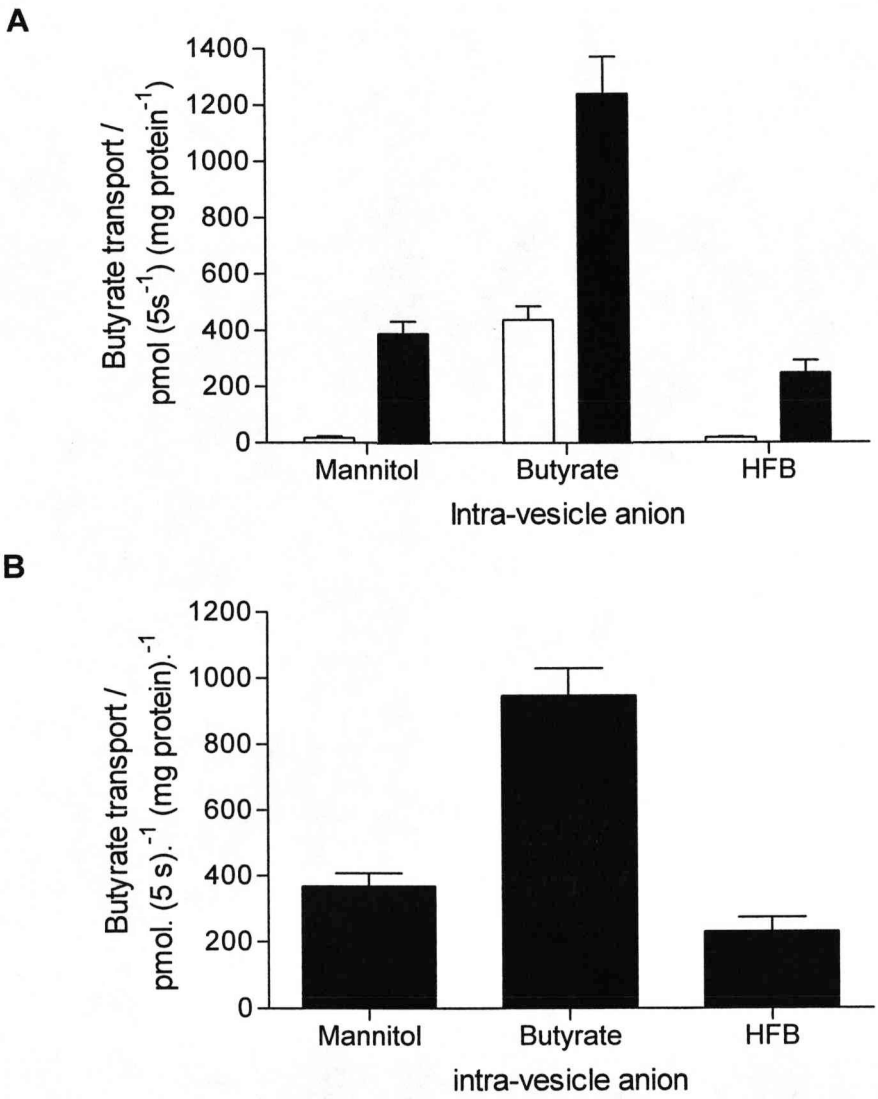


Figure 5.4 Butyrate/butyrate exchange across porcine colonic LMVs

Porcine colonic LMV were loaded with a buffer containing 20 mM Hepes/tris pH 7.5, 0.1 mM $MgSO_4$ and 300 mM mannitol or 100 mM mannitol with 100 mM sodium butyrate or sodium HFB. LMV (50 μg protein per assay) were incubated in a buffer containing either 20 mM Hepes/tris pH 7.5 (□) or Mes/tris pH 5.5 (■) and 0.1 mM $MgSO_4$ and 1 mM sodium butyrate for 5 seconds. Uptake was carried out at 37 °C for 5 s as described in Methods (section 2.8). **A**, butyrate up at pH_{out} 5.5 and 7.5; **B**, pH stimulated butyrate uptake as determined by subtracting the uptake at pH 7.5 from the uptake at pH 5.5. Rates are expressed as mean ± S.E.M. 3 separate vesicle preparations.

In order to establish a clear picture of butyrate/butyrate exchange versus HFB/butyrate exchange the pH stimulated butyrate uptake was calculated. This was determined by subtracting the uptake at pH_{out} 7.5 from the uptake at pH_{out} 5.5. As seen in figure 5.4b butyrate/HFB exchange had a rate of 230.3 ± 42.7 pmoles/5 s/mg protein; a rate significantly smaller than the rate observed for butyrate/butyrate exchange which had a rate of 946.8 ± 83.21 pmoles/5 s/mg protein, and a smaller rate then butyrate/hydroxyl (368.4 ± 40.13 pmoles/5 s/mg protein). The data indicated that HFB can directly exchange with butyrate, indicating that it is transported by the butyrate transporter but having possibly a lower affinity for the transporter compared to butyrate.

5.2.3.3 Inhibition of butyrate uptake by HFB

After determining that HFB is able to exchange with butyrate and be transported out of colonic LMVs, albeit at a lower rate, we next sought to determine if HFB could inhibit butyrate transport into the LMVs. Colonic LMVs were preloaded with 300 mM mannitol, 20 mM Hepes/tris pH 7.5 and 0.1 mM $MgSO_4$. Uptake of butyrate was carried out in an iso-osmolar buffer consisting of 100 mM mannitol, 100 mM Na gluconate, 20 mM Mes/tris pH 5.5, 1 mM Na butyrate and 0.1 mM $MgSO_4$ ± 5 mM NaHFB. Uptake of butyrate without addition of 5 mM NaHFB gave a rate of 385.7 ± 44.14 pmoles/5 s/mg protein, which is in agreement with the rate obtained previously (see section 5.2.3.1). When the rate of butyrate uptake was determined in the presence of 5 mM HFB to the uptake medium, a rate of 194.8 ± 31.43 pmoles/5 s/mg protein was observed; a reduced rate of 49 %. This clearly shows that HFB can inhibit butyrate transport into porcine colonic LMV implying that HFB binds to the butyrate transporter.

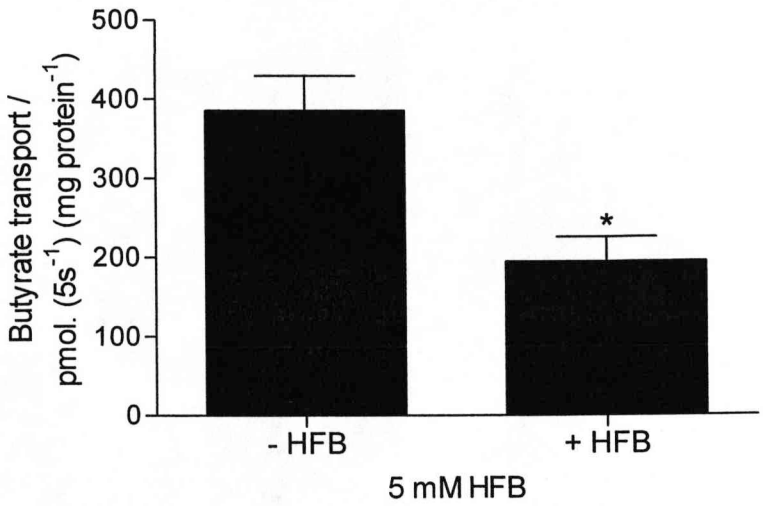


Figure 5.5 HFB Inhibition of butyrate transport

Porcine colonic LMV were loaded with a buffer containing 300 mM mannitol, 20 mM hepes/tris pH 7.5 and 0.1 mM $MgSO_4$. LMV (50 μg protein per assay) were incubated in a buffer containing 100 mM mannitol, 100 mM Na glcuonate, 20 mM Mes/tris pH 5.5, 0.1 mM $MgSO_4$ and 1 mM sodium butyrate ± 5 mM sodium HFB. Uptake took place at 37 °C for 5 s as described in Methods (section 2.8). Rates are expressed as mean ± S.E.M. 3 separate vesicle preparations (* = p-vaule ≤ 0.05).

5.2.4 Treatment of colonic epithelial cell lines with sodium HFB

After determining that HFB was transported into porcine colonic LMVs, we next set out to determine if HFB was able to mimic butyrates effects on gene expression; therefore assessing if some butyrate regulated gene expression may be responsive to butyrate metabolism. Two cell lines, a non-tumourigenic AA/C1, adenoma-derived cell line, and a HT-29, adenocarcinoma-derived cell line were used. These cells express MCT1 on their luminal membrane (Cuff *et al.*, 2002; Lambert *et al.*, 2002; Rizhaupt *et al.*, 1998a) and it has been shown previously that the properties of butyrate transport in these cells is similar to that in porcine and human colonic luminal membrane vesicles (Rizhaupt *et al.*, 1998a). After reaching the desired confluency the cell lines were treated with 5 mM NaBut and NaHFB for 24 hours, after which time the cells were harvested and total RNA isolated. Total cDNA was prepared and the expression of the butyrate-responsive genes p21, IAP, Bak and Bcl-x_L mRNA was determined in both cell lines, while the expression of survivin and MCT1 mRNA was only determined in AA/C1 cells. The changes in the expression of the butyrate-responsive genes measured by real-time PCR are listed in table 5.2. Treatment of HT-29 cells with NaHFB resulted in no change in the expression of IAP or Bak compared to the untreated control. HFB treatment did result in a slight 1.1 fold increase in p21 mRNA expression; however this increase was far less than that observed in response to butyrate treatment (4.20 fold increase in p21 mRNA expression seen after butyrate treatment). NaHFB treatment of HT-29 cells resulted in a 1.3 fold increase in Bcl-x_L mRNA expression, compared to the 1.7 fold decrease seen in Bcl-x_L mRNA expression after butyrate treatment.

Table 5.2 Changes in mRNA abundance in response to 5 mM NaBut and NaHFB

	HT-29		AA/C1	
gene	NaBut	NaHFB	NaBut	NaHFB
p21	4.196 ± 0.03 ↑	1.148 ± 0.05	1.406 ± 0.112 ↑	0.957 ± 0.081
IAP	29.174 ± 4.655 ↑	1.056 ± 0.215	2.060 ± 0.089 ↑	1.060 ± 0.090
Bak	2.001 ± 0.152 ↑	1.017 ± 0.095	1.849 ± 0.09 ↑	0.959 ± 0.164
Bcl-x_L	0.590 ± 0.080 ↓	1.251 ± 0.081	0.694 ± 0.09 ↓	0.967 ± 0.089
Birc5	*n.d.*	*n.d.*	0.391 ± 0.024 ↓	0.966 ± 0.037
MCT1	*n.d.*	*n.d.*	2.035 ± 0.139 ↑	0.967 ± 0.037

HT-29 cells and AA/C1 cells were grown to 70 and 100 % confluency respectively and either left untreated or treated with 5 mM NaBut or 5 mM NaHFB for 24 hours. After the appropriate time, total RNA was isolated and cDNA was prepared. The abundance of the mRNA for genes listed was measured using real-time PCR. Results are expressed as arbitrary units relative to an untreated control equal to 1 unit, ± S.E.M. of three separate cell treatments. *n.d.* not determined. Arrows indicate an increase (↑), or decrease (↓) in gene expression

5.3 Discussion

Porcine colonic LMVs were prepared using the cation-precipitation and differential centrifugation techniques from pig colonic mucosal scrapings. They were then characterised to assess the enrichment and recovery of the luminal, basolateral and organelle marker proteins. The results indicated negligible presence of organelles and basolateral membranes in the luminal membrane vesicles. Cysteine-sensitive alkaline phosphatase, a marker for the luminal membrane (Vengesa & Hopfer, 1979; Harig *et al.*, 1990; Rizthaut *et al.*, 1998a) was used to assess the origin of the LMVs. Cysteine-sensitive AP was enriched 6.99 ± 1.02 over the cell homogenate, with a recovery of 23.25 ± 3.59 %, in agreement with enrichment and recoveries previously reported for colonic LMV preparations (Harig *et al.*, 1990; Ritzhaupt, 1998a; Vengesa & Hopfer, 1979). Furthermore, the colonic LMVs were assessed using western blotting to determine the abundance of marker proteins in the luminal and basolateral membranes. Villin and MCT1, markers of the luminal membrane and HLA-1, a marker of the basolateral membrane were assessed. Villin and MCT1 were enriched 2 and 4 fold respectively; while HLA-1 was disenriched 3 fold in the LMVs. The enrichment of villin in the LMV fraction is small compared to brush border membrane vesicles produced from the small intestine. Small intestinal enterocytes have more abundant microvilli compared to colonocytes, which corresponds to a higher abundance of villin (Dudouet *et al.*, 1987). The microvilli of the enterocytes are also more tightly compacted allowing a higher recovery of the brush border member. The enzyme assays and immunoblots for membrane markers, showed high enrichment for the markers of the luminal membrane and negligible contamination of basolateral and organelle membranes in the LMVs.

As there is no ^{14}C radiolabelled HFB tracer commercially available, a different approach was used to investigate HFB transport into colonic LMVs. Butyrate is transported into the colonocytes using a pH dependent anion exchange process. Butyrate can be taken up into LMVs in exchange for intravesicular butyrate in the presence of a pH gradient. This trans-stimulation of butyrate allows studying the transport of butyrate/butyrate exchange from the inside to outside of the vesicle. Pre-loading the LMVs with HFB allowed an exchange between extravesicular butyrate and the intravesicular HFB to take place. The influx of the radiolabelled butyrate tracer was dependent on the efflux of the HFB, therefore allowing HFB transport to be studied indirectly. Transport of HFB via butyrate/HFB exchange was determined to be significantly lower than that for butyrate/butyrate exchange. Indeed, the transport rates obtained for HFB/butyrate exchange was slightly lower than butyrate/hydroxyl exchange. However, some transport of HFB did take place. Furthermore, this transport was stimulated at pH 5.5, a characteristic of butyrate transport into colonic LMVs (Rizhaupt, 1998a).

After determining that HFB is transported into porcine colonic LMVs we next set out to determine if butyrate metabolism plays a role in the regulation of some butyrate responsive genes. HFB failed to modulate the expression of p21, IAP, Bak, Bcl-x_L, survivin and MCT1 in AA/C1 cells and IAP and Bak in HT-29 cells. A slight up-regulation of p21 and bcl-x_L was seen when HT-29 cells were treated with HFB. The slight up-regulation of p21 and Bcl-x_L in HT-29 cells by HFB is probably coincidental. This conclusion is made due to the fact that butyrate down-regulated Bcl-x_L in both cell lines while HFB treatment resulted in up-regulation of Bcl-x_L in only one cell line. The modulation of p21 expression seen in HFB-treated HT-29 cells is not seen in HFB-treated AA/C1

cells compared to the modulation of p21 expression by butyrate, observed in both cell lines. Furthermore, as discussed in chapter 4, there is evidence that Bak and survivin as well as Bcl-x_L are regulated by butyrate through a potential luminal membrane butyrate sensor(s). If HFB was able to mimic the effects of butyrate, via the putative butyrate sensor, one would expect HFB to interact with the extra-cellular sensor(s), resulting in the modulation of Bak, survivin and Bcl-x_L.

The lack of effect of HFB can be interpreted in two ways; 1) that all the genes tested require butyrate metabolism for their regulation, 2) HFB is structurally different to butyrate and is unable to mimic any of butyrates effects. The van der waal's radius of the fluorine atom (1.35 Å) is close to that of hydrogen (1.1 Å) (Goldman, 1969), but is still larger. The difference in the chemical structure of butyrate and HFB may be sufficient for the lack of the effect.

3.3.1 Intracellular mechanisms involved in butyrate regulation of gene expression

Nutrients are known to be sensed intra-cellularly by binding to transcription factors within the nucleus. These transcription factors act as nutrient sensors by changing the level of DNA transcription. During ligand binding, these nuclear nutrient receptors undergo conformational changes that result in coordinated dissociation of co-repressor and recruitment of co-activator proteins. The transcription factors bind to specific nucleotide sequences in the promoter regions of a gene, known as response elements, to enable transcription activation (Afman & Muller, 2006). Butyrate is known to up-regulate the expression of numerous genes, many of which involve up-

regulation of transcription. The activation of transcription by butyrate could involve butyrate alone or one or more of the downstream products of butyrate metabolism.

As shown in figure 5.6, butyrate undergoes metabolism by a complex pathway involving numerous enzymes. Silencing any one or a number of these enzymes would prevent the metabolism of butyrate and therefore the regulation of any gene that required a downstream metabolite of butyrate would be prevented. Targeting the machinery by which butyrate is metabolised is another route to discovering those genes that are dependent on butyrate metabolism.

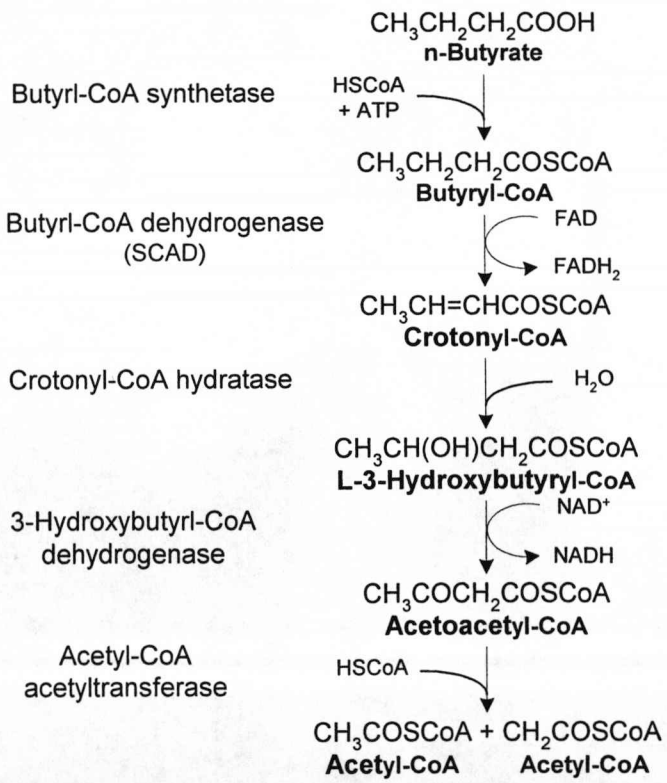


Figure 5.6 Metabolism of butyrate in colonocytes

Adapted from Roediger, (1994)

5.4 General discussion and future directions

Butyrate is a four carbon small chain monocarboxylate that is produced in the colon by the resident microflora through fermentation of dietary fibre and resistant starch. Butyrate plays an important role in maintaining colonic tissue homeostasis. Butyrate is the principle energy source for the colonocytes and has been shown to be responsible for the regulation of numerous genes associated with proliferation, apoptosis and differentiation. (Tan *et al.*, 2002; Daly & Shirazi-Beechey, 2006). In many cases these genes have shown to be deregulated during colorectal tumorigenesis (Tabuchi *et al.*, 2006). In order to understand the role butyrate plays in the regulation of the butyrate responsive target genes, how and why they are deregulated in CRC, one must first completely understand how butyrate modulates gene expression.

The mechanism by which butyrate regulates gene expression can be divided into two clear routes: 1) Butyrate-induced gene expression initiated by entry of butyrate into the colocytes; 2) Butyrate-induced gene expression initiated through the interaction of butyrate with the colonic luminal membrane butyrate receptor. Route one can be further subdivided into pathways that are directly affected by butyrate itself, such as its role as a histone deactylase inhibitor (Hague & Paraskeva, 1995); and others that are influenced by a metabolite of butyrate metabolism. Genes such as *bak1*, *bcl-*x_L and *BIRC5* appear to be regulated via a luminal membrane "butyrate receptor" such as GRP41 or 43. Whereas, the regulation of genes such as *ALP1*, *p21* and *mct1* are dependent on the entry of butyrate into the colocytes. However, whether the regulation of these genes is also dependent on butyrate metabolism is yet to be determined.

The work presented in this thesis shows the successful synthesis of disodium $PEG_{600}\alpha,\omega$-di(4-butyrate). However, the problems faced during purification resulted in a small yield being obtained. As mentioned previously, disodium $PEG_{600}\alpha,\omega$-di(4-butyrate) can be utilised to identify further genes that do not require butyrate entry into the cell and may be regulated by the putative butyrate sensor. For the production of disodium $PEG_{600}\alpha,\omega$-di(4-butyrate) on a wide ranging scale, the synthesis of disodium $PEG_{600}\alpha,\omega$-di(4-butyrate) may have to be further modified to increase the yield.

Investigations into the effect on butyrate metabolism using HFB did not yield any concrete data on the effectiveness of HFB as a non-metabolisable butyrate analogue. The failure of HFB to induce apoptosis in HT-29 and SW620 colon adenocarcinoma cell lines led Heerdt *at al.*, (1994) to propose that butyrate-induced apoptosis was dependent on the metabolism of butyrate. However, the authors did not show any positive regulation by HFB, i.e. no genes were shown to be modulated, either positively or negatively, by HFB.
As discussed in chapter 5, HFB did not modulate the expression of bak in either AA/C1 or HT-29 cell lines. It is proposed in this thesis that bak is modulated by butyrate through an extra-cellular sensor located on the luminal membrane of the colonocyte. In order for HFB to be classified as a non-metabolisable butyrate analogue, it is assumed that HFB is able to mimic any effects of butyrate that do not require the metabolism of butyrate; the modulation of bak expression by butyrate, through an extra-cellular sensor does not require butyrate metabolism. It was expected that HFB would modulate the expression of bak, mimicking butyrate. However, this is not the case.

The lack of a positive control for HFB treatment *in vitro* and its failure to modulate the expression of bak, brings doubt on the appropriateness of HFB as a non-metabolisable analogue. This must be considered in the future for any use of HFB as a non-metabolisable butyrate-analogue.

Work on-going in our laboratory is directed towards silencing the expression of various enzymes responsible for the metabolism of butyrate and the proteins responsible for the transport of butyrate-CoA into the mitochondria. Silencing the machinery responsible for the metabolism of butyrate would identify those genes that were regulated by a butyrate metabolite at that point on wards.

On-going work is also focused on identifying the putative butyrate sensor through silencing the G-protein coupled receptors GPR41 and GPR43. Once identified, the downstream pathway activated by the butyrate sensor can be investigated, allowing the molecular mechanisms by which butyrate modulates gene expression to be further clarified. Further investigation is required to clarify the positive-modulation of bcl-x_L seen when AA/C1 and HT-29 cell lines are treated with PRG-ba. As discussed in chapter 4, PEG-ba significantly up-regulates bcl-x_L; this being in stark contrast to the down-regulation of bcl-x_L seen when AA/C1 and HT-29 cell lines are treated with butyrate. It is hoped that co-treatment of butyrate and PEG-ba to AA/C1 and HT-29 cell lines will help to resolve the issue.

Further work is required to identify the mechanism by which butyrate regulates gene expression. The work presented here is a small step towards our greater understanding of these butyrate responsive regulatory pathways and their deregulation during the development of colorectal cancer.

CHAPTER 6

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